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<b>(21) International Application Number:</b> PCT/US96/10251 <b>(22) International Filing Date:</b> 6 June 1996 (06.06.96) <b>(30) Priority Data:</b> 08/480,473 6 June 1995 (06.06.95) US <b>(71) Applicant:</b> THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US). <b>(72) Inventor:</b> SEMENZA, Gregg, L.; 718 Scarlett Drive, Towson, MD 21286 (US). <b>(74) Agent:</b> HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE  <b>(57) Abstract</b> <p>The purified and characterization of hypoxia-inducible factor 1 (HIF-1) is described. HIF-1 is composed of subunits HIF-1<math>\alpha</math> and HIF-1<math>\beta</math>. Purified HIF-1<math>\alpha</math> polypeptide, its amino acid sequence and polynucleotide sequence are provided. A HIF-1<math>\alpha</math> variant that dimerizes to HIF-1<math>\beta</math> producing a nonfunctional HIF-1 complex is described. Methods for the prevention and treatment of hypoxia-related disorders are provided.</p>		

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## HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE

### Statement as to Federally Sponsored Research

5 This invention was made in part with funds from the Federal government, PHS grant RO1-DK39869. The government therefore has certain rights in the invention.

### FIELD OF THE INVENTION

This invention relates to hypoxia-related proteins, and specifically to novel DNA-binding proteins which are induced by hypoxia.

### Background of the Invention

10 Mammals require molecular oxygen ( $O_2$ ) for essential metabolic processes including oxidative phosphorylation in which  $O_2$  serves as electron acceptor during ATP formation. Systemic, local, and intracellular homeostatic responses elicited by hypoxia (the state in which  $O_2$  demand exceeds supply) include erythropoiesis  
15 by individuals who are anemic or at high altitude (Jelkmann (1992) *Physiol. Rev.* 72:449-489), neovascularization in ischemic myocardium (White et al. (1992) *Circ. Res.* 71:1490-1500), and glycolysis in cells cultured at reduced  $O_2$  tension (Wolfe et al. (1983) *Eur. J. Biochem.* 135:405-412). These adaptive responses either increase  $O_2$  delivery or activate alternate metabolic pathways that do not require  
20  $O_2$ . Hypoxia-inducible gene products that participate in these responses include erythropoietin (EPO) (reviewed in Semenza (1994) *Hematol. Oncol. Clinics N. Amer.* 8:863-884), vascular endothelial growth factor (Shweiki et al. (1992) *Nature* 359:843-845; Banai et al. (1994) *Cardiovasc. Res.* 28:1176-1179; Goldberg & Schneider (1994) *J. Biol. Chem.* 269:4355-4359), and glycolytic enzymes (Firth et  
25 al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500; Semenza et al. (1994) *J. Biol. Chem.* 269:23757-23763).

The molecular mechanisms that mediate genetic responses to hypoxia have been extensively investigated for the EPO gene, which encodes a growth factor that regulates erythropoiesis and thus blood  $O_2$ -carrying capacity (Jelkmann  
30 (1992) *supra*; Semenza (1994) *supra*). *Cis*-acting DNA sequences required for transcriptional activation in response to hypoxia were identified in the EPO 3'-flanking region and a *trans*-acting factor that binds to the enhancer,

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hypoxia-inducible factor 1 (HIF-1), fulfilled criteria for a physiological regulator of EPO transcription: inducers of EPO expression (1% O<sub>2</sub>, cobalt chloride [CoCl<sub>2</sub>], and desferrioxamine [DFX]) also induced HIF-1 DNA binding activity with similar kinetics; inhibitors of EPO expression (actinomycin D, cycloheximide, and 2-aminopurine) blocked induction of HIF-1 activity; and mutations in the EPO 3'-flanking region that eliminated HIF-1 binding also eliminated enhancer function (Semenza (1994) supra). These results also support the hypothesis that O<sub>2</sub> tension is sensed by a hemoprotein (Goldberg et al. (1988) Science 242:1412-1415) and that a signal transduction pathway requiring ongoing transcription, translation, and protein phosphorylation participates in the induction of HIF-1 DNA-binding activity and EPO transcription in hypoxic cells (Semenza (1994) supra).

EPO expression is cell type specific, but induction of HIF-1 activity by 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX was detected in many mammalian cell lines (Wang & Semenza (1993a) Proc. Natl. Acad. Sci. USA 90:4304-4308), and the EPO enhancer directed hypoxia-inducible transcription of reporter genes transfected into non-EPO-producing cells (Wang & Semenza (1993a) supra; Maxwell et al. (1993) Proc. Natl. Acad. Sci. USA 90:2423-2427). RNAs encoding several glycolytic enzymes were induced by 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX in EPO-producing Hep3B or non-producing HeLa cells whereas cycloheximide blocked their induction and glycolytic gene sequences containing HIF-1 binding sites mediated hypoxia-inducible transcription in transfection assays (Firth et al. (1994) supra; Semenza et al. (1994) supra). These experiments support the role of HIF-1 in activating homeostatic responses to hypoxia.

## SUMMARY OF THE INVENTION

The invention features a substantially purified DNA-binding protein, hypoxia-inducible factor-1 (HIF-1), characterized as activating structural gene expression where the promoter region of the structural gene contains an HIF-1 binding site. Examples of such structural genes include erythropoietin (EPO), vascular endothelial growth hormone (V-EGF), and glycolytic genes. HIF-1 is composed of two subunits, HIF-1 $\alpha$  and an isoform of HIF-1 $\beta$ .

The invention features a substantially purified HIF-1 $\alpha$  polypeptide, and a nucleotide sequence which encodes HIF-1 $\alpha$ .



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The invention provides methods for preventing and treating hypoxia-related disorders, including tissue damage resulting from hypoxia and reperfusion, by administering a therapeutically effective amount of HIF-1 protein. Also included in the invention is gene therapy by introducing into cells a nucleotide sequence  
5 encoding HIF-1. The invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier admixed with a therapeutically effective amount of HIF-1 or nucleotide sequence encoding HIF-1.

The invention further provides a novel HIF-1 $\alpha$  variant polypeptide which functionally inactivates HIF-1 *in vivo*. The invention provides a method for treating  
10 an HIF-1-mediated disorder or condition by functional inactivation of HIF-1 by administration of an effective amount of the HIF-1 $\alpha$  variant of the invention.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 is a autoradiograph showing dose-dependent induction of HIF-1 DNA binding activity by CoCl<sub>2</sub> treatment. Nuclear extracts, prepared from HeLa cells.  
15 cultured in the presence of the 0, 5, 10, 25, 50, 75, 100, 250, 500, or 1000  $\mu$ M of CoCl<sub>2</sub> for 4 h at 37°C, were incubated with W18 probe and analyzed by gel shift assay. Lanes 1-8 and 9-12 represent extracts prepared in two separate experiments. Arrows indicate HIF-1, constitutive DNA binding activity (C), nonspecific activity (NS), and free probe (F).

Fig. 2 is an autoradiograph showing the results of methylation interference analysis with nuclear extracts from CoCl<sub>2</sub>-treated HeLa cells. W18 was 5'-end labeled on the coding or noncoding strand, partially methylated, and incubated with nuclear extracts. DNA-protein complexes corresponding to HIF-1, constitutive DNA binding activities (C1 and C2), and nonspecific binding activity (NS) were isolated from a preparative gel shift assay (lower) in addition to free  
25 probe (F) (not shown). DNA was purified, cleaved with piperidine, and analyzed on a 15% denaturing polyacrylamide gel (upper). Results are summarized at left for coding strand and at right for noncoding strand. The guanine residues are numbered according to their locations on the W18 probe. The HIF-1 binding site is boxed. Complete methylation interference with HIF-1 binding is indicated in  
30 closed circles; partial and complete methylation interference with constitutive DNA binding activity are indicated by open and closed squares, respectively.

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Fig. 3A is an autoradiograph showing gel shift assay analysis of column fractions for HIF-1 DNA binding activity. Nuclear extracts were fractionated by DEAE-Sepharose chromatography, and fractions containing HIF-1 activity were applied to a W18 DNA affinity column. 5 ug of protein were incubated with 0.1 ug of calf thymus DNA for gel shift analysis of crude nuclear extract (Crude NE, lane 1) and HIF-1 active fractions from DEAE-Sepharose columns (DEAE, lane 2). For fractions from the W18 column (lanes 3-13), 1 ul aliquots were incubated with 5 ng of calf thymus DNA. The positions of the two HIF-1 bands, constitutive activity (C), nonspecific activity (NS), and free probe (F) are indicated. FT, flowthrough, 0.25 M, 0.5 M, 1 M, and 2 M are fractions eluted with indicated concentration of KCl in buffer Z.

Fig. 3B is an autoradiograph showing sequence-specific DNA binding of the partially purified fractions described in the legend to Fig. 3A. 5 ug aliquots of fractions from the DEAE-Sepharose column were incubated with W18 probe in the presence of no competitor (lane 1), 10-fold (lanes 2 and 5), 50-fold (lanes 3 and 6), or 250-fold (lanes 4 and 7) molar excess of unlabeled W18 (W, lanes 2-4) or M18 (M, lanes 5-7) oligonucleotide.

Fig. 4A is an autoradiograph showing purification of HIF-1 from CoCl<sub>2</sub>-treated HeLa S3 cells. Flowthrough fraction from the M18 DNA column (Load, lane 1) and 0.25 M KCl and 0.5 M KCl fractions from the second W18 DNA affinity column (lanes 2 and 3) were analyzed. An aliquot of each fraction (5 ug of load or 1 ug of affinity column fractions) were resolved by 6% SDS-PAGE and silver stained. HIF-1 polypeptides in lanes 2 and 3 are indicated by arrows at the right of the figure.

Fig. 4B is an autoradiograph showing HIF-1 purification from hypoxic Hep3B cells. HIF-1 fractions from the first W18 column (Load, lane 1) and 0.25 M KCl and 0.5 M KCl fractions from the second W18 column (lanes 2 and 3) were analyzed. An aliquot of each fraction (50 ul) was resolved by 7% SDS-PAGE and silver stained. Molecular mass markers are myosin (200 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), and ovalbumin (45 kDa). HIF-1 polypeptides in lanes 2 and 3 are indicated by arrows at the right of the figure.

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Fig. 5A is an autoradiograph identifying the HIF-1 polypeptides. An aliquot of affinity-purified HIF-1 was resolved on a 6% SDS-polyacrylamide gel with 3.2% cross-linking along with the HIF-1 protein complex isolated by preparative native gel shift assay (HIF-1). MW, molecular mass markers with size (kDa) indicated at left of figure; numbers to the right of figure indicate the apparent molecular weights (kDa) of HIF-1 polypeptides.

Fig. 5B is an autoradiograph showing the HIF-1 components on a 6% SDS-polyacrylamide gel with 5% cross-linking. An aliquot of affinity-purified HIF-1 was resolved on a 6% SDS-polyacrylamide gel along with the HIF-1 protein complex isolated by preparative native gel shift assay (HIF-1). The 120 kDa polypeptide, 94/93/91 kDa polypeptides, and two contaminant proteins (\*1 and \*2) are indicated.

Fig. 5C is an autoradiograph showing the alignment of HIF-1 components identified on two gel systems with different degrees of cross-linking. Gel slices isolated from the 6% SDS-polyacrylamide gel with 5% cross-linking corresponding to 120 kDa HIF-1 polypeptide (12), 94/93/91 kDa HIF-1 polypeptide (94/93/91), and two contaminant proteins (\*1 and \*2) were resolved on a 6% SDS-polyacrylamide gel with 3.2% cross-linking in parallel with an aliquot (30 ul) of affinity purified HIF-1 (Fig. 5A).

Fig. 6 is a graph of the absorbance profiles at 215 nm of tryptic peptides derived from 91 kDa HIF-1 polypeptide (top), 93/94 kDa polypeptides (middle), and trypsin (bottom).

Fig. 7 is an autoradiograph showing UV cross-linking analysis with affinity purified HIF-1 and probe W18 in the absence (lane 1) or presence of 250-fold molar excess of unlabeled W18 (lane 2) or M18 (lane 3) oligonucleotide. The binding reaction mixtures were UV-irradiated and analyzed on a 6% SDS-polyacrylamide gel. Molecular mass standards are indicated at left.

Fig. 8 is an autoradiograph showing the results of glycerol gradient sedimentation analysis. Nuclear extracts prepared from Hep3B cells exposed to

1% O<sub>2</sub> for 4 h (Load) was sedimented through a 10-30% linear glycerol gradient. Aliquots (10 ul) from each fraction were analyzed by gel shift assay. Arrows at top indicate the peak migration for ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa).

5           FIG. 9 is a diagram of the cDNA sequence encoding HIF-1 $\alpha$ . Bold lines indicate extent of clones hbc120, hbc025, and 3.2-3 relative to the full-length RNA-coding sequence shown below. Box, amino acid coding sequences; thin line, untranslated sequences; bHLH, basic helix-loop-helix domain; A and B, internal homology units within the PAS domain.

10           Fig. 10 is the nucleotide and derived amino acid sequence of HIF-1 $\alpha$ . A composite sequence was derived from the complete nucleotide sequences determined for clones 3.2-3 (nt 1-3389), hbc025 (nt 135-3691), and hbc120 (nt 1739-3720). Sequences of four tryptic peptides obtained from the purified HIF-1 $\alpha$  120 kDa polypeptide are underscored (two peptides are contiguous).

15           Fig. 11 is the analysis of bHLH domains. Coordinate of first residue of each sequence and amino acid identity with HIF- 1 $\alpha$  or HIF- 1 $\beta$  (ARNT) are given in parentheses at left and right margins, respectively. Hyphen indicates gap introduced into sequence to maximize alignment except in consensus where it indicates a lack of agreement. Consensus indicates at least 3 proteins with  
20 identical or similar residue at a given position. 1: F, I, L, M, or V; 2: S or T; 3: D or E; 4: K or R. Invariant residues are shown in bold.

25           Fig. 12 is the analysis of PAS domains. Alignments of PAS A (top) and B (bottom) subdomains are shown. Consensus indicates at least 4 proteins with identical or similar residue at a given position. GenBank accession numbers: ARNT, M69238; AHR, L19872; SIM, M19020; MI, Z23066; USF, X55666; L-MYC, X13945; CP-1, M34070; PER, M30114; KinA, M31067.

Fig. 13A is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 1% O<sub>2</sub> for 0, 1, 2, 4, 8, and 16 h.

Fig. 13B is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 75  $\mu$ M CoCl<sub>2</sub> for 0, 1, 2, 4, 8, and 16 h.

Fig. 13C is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 130  $\mu$ M desferrioxamine (DFX) for 0, 1, 2, 4, 8, and 16 h.

Fig. 13D is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposing Hep3B cells to 1% O<sub>2</sub> for 4 h, then returning the cells to 20% O<sub>2</sub> for 0, 5, 15, 30, or 60 min prior to RNA isolation.

Fig. 13E is a table of the AUUUA-containing elements from the HIF-1 $\alpha$  3'-UTR. The first nucleotide is numbered according to the composite cDNA sequence.

Fig. 14A is an autoradiograph of nuclear extracts from hypoxic Hep3B cells incubated with oligonucleotide probe W18 for 10 min on ice, immune sera was added (lanes 2 and 5) and incubated for 20 min on ice, followed by polyacrylamide gel electrophoresis. Preimmune sera (lanes 3 and 5) and antisera (lanes 2 and 4) were obtained from rabbits before and after immunization, respectively, with GST/HIF-1 $\alpha$  (lanes 2 and 3) or GST/HIF-1 $\beta$  (lanes 4 and 5). HIF-1, constitutive (C) and nonspecific (NS) DNA binding activities, free probe (F), and supershifted HIF-1/DNA/antibody complex (S) are indicated.

Fig. 14B is an immunoblot showing antisera recognition of HIF-1 subunits present in purified protein preparations and crude protein extracts. Nuclear extracts from Hep3B cells which were untreated (lane 1) or exposed to 1% O<sub>2</sub> for 4 h (lane 2) and from HeLa cells which were untreated (lane 6) or exposed to 75  $\mu$ M CoCl<sub>2</sub> for 4 h (lane 7) were fractionated on a 6% SDS/polyacrylamide gel in parallel with 1, 2, and 5  $\mu$ l of affinity-purified HIF-1 from CoCl<sub>2</sub>-treated HeLa cells (lanes 3-5). Protein was transferred to a nitrocellulose membrane and incubated with antisera to HIF-1 $\alpha$  (top) or HIF-1 $\beta$  (bottom).

Fig. 14C is an immunoblot showing the induction kinetics of HIF-1 $\alpha$  and HIF-1 $\beta$  protein in hypoxic cells. Hep3B cells were exposed to 1% O<sub>2</sub> for 0 to 16 h prior to preparation of nuclear (N.E.) and cytoplasmic (C.E.) extracts, and immunoblot analysis was performed with antisera to HIF-1 $\alpha$  (top) or HIF-1 $\beta$  (bottom).

5 Fig. 14D is an immunoblot showing decay kinetics of HIF-1 $\alpha$  and HIF-1 $\beta$  polypeptides in post-hypoxic cells. Hep3B cells were exposed to 1% O<sub>2</sub> for 4 h and returned to 20% O<sub>2</sub> for 0 to 60 min prior to preparation of extracts and immunoblot analysis. Arrowheads distinguish HIF-1 subunits from cross-reacting proteins of unknown identity.

10 Fig. 15A is an diagram of the structure of reporter gene constructs used for functional analysis of HIF-1 binding sites in human aldolase A (hALDA), human phosphoglycerate kinase 1 (hPGK1), and mouse phosphofructokinase L (mPFKL) genes. Arrow, transcription initiation site; box, hEPO 3'-FS (cross-hatched), hPGK1 5'-FS (stippled), or mPFKL IVS-1 (striped) oligonucleotide (sequences are  
15 as shown in Table 3). DNA fragments from the 5'-end of the hALDA gene in pNMHcat and pHcat are 3.5 and 0.76 kb, respectively, and are colinear at the 3'-end where they are directly fused to CAT coding sequences.

20 Fig. 15B is a bar graph showing CAT/ $\beta$ -galactosidase expression (relative CAT activity) in transfected cells exposed to 20% O<sub>2</sub> (open bar) or 1% O<sub>2</sub> (closed bar). Data are plotted using lower scale for all results except those for pHcat, which are plotted according to the upper scale. Induction, representing the relative CAT activity at 1% O<sub>2</sub>/20%O<sub>2</sub>, was calculated for each experiment; mean and standard error of mean (SEM) were determined for results from n independent experiments.

25 Fig. 16 is the amino-terminal (top) and carboxy-terminal (bottom) amino acid sequence of the wild-type and dominant-negative variant forms of HIF-1 $\alpha$ .

#### **DETAILED DESCRIPTION OF THE INVENTION**

30 The invention provides a substantially pure hypoxia-inducible factor-1 (HIF-1) characterized as a DNA-binding protein which binds to a region in the regulatory, preferably in the enhancer region, of a structural gene having the HIF-1 binding

motif. Included among the structural genes which can be activated by HIF-1 are erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic gene transcription in cells subjected to hypoxia. Analysis of purified HIF-1 shows that it is composed of subunits HIF-1 $\alpha$  and an isoform of HIF-1 $\beta$ . In addition to  
5 having domains which allow for their mutual association in forming HIF-1, the  $\alpha$  and  $\beta$  subunits of HIF-1 both contain DNA-binding domains. The alpha subunit is uniquely present in HIF-1, whereas the beta subunit (ARNT) is a component of at least two other transcription factors.

The invention provides a substantially pure hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) polypeptide characterized as having a molecular weight of 120 kDa as  
10 determined by SDS-PAGE and having essentially the amino acid sequence of SEQ ID NO:2 (Fig. 10) and dimerizing to HIF-1 $\beta$  to form HIF-1. The term "substantially pure" as used herein refers to HIF-1 $\alpha$  which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally  
15 associated. One skilled in the art can purify HIF-1 $\alpha$  using standard techniques for protein purification. The substantially pure polypeptide will yield a single band on a non-reducing polyacrylamide gel. The purity of the HIF-1 $\alpha$  polypeptide can also be determined by amino-terminal amino acid sequence analysis. HIF-1 $\alpha$  protein includes functional fragments of the polypeptide, as long as the activity of HIF-1 $\alpha$ ,  
20 such as the ability to bind with HIF-1 $\beta$ , remains. Smaller peptides containing the biological activity of HIF-1 $\alpha$  are included in the invention.

The invention provides nucleotide sequences encoding the HIF-1 $\alpha$  polypeptide (SEQ ID NO:1)(Fig. 10). These nucleotides include DNA, cDNA, and RNA sequences which encode HIF-1 $\alpha$ . It is also understood that all nucleotide  
25 sequences encoding all or a portion of HIF-1 $\alpha$  are also included herein, as long as they encode a polypeptide with HIF-1 $\alpha$  activity. Such nucleotide sequences include naturally occurring, synthetic, and intentionally manipulated nucleotide sequences. For example, HIF-1 $\alpha$  nucleotide sequences may be subjected to site-directed mutagenesis. The nucleotide sequence for HIF-1 $\alpha$  also includes  
30 antisense sequences. The nucleotide sequences of the invention include sequences that are degenerate as a result of the genetic code. All degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of HIF-1 $\alpha$  polypeptide which is encoded by the nucleotide sequence is functionally unchanged.

Specifically disclosed herein is a DNA sequence encoding the human HIF-1 $\alpha$  gene. The sequence contains an open reading frame encoding a polypeptide 826 amino acids in length. The human HIF-1 $\alpha$  initiation methionine codon shown in FIG. 10 at nucleotide position 29-31 is the first ATG codon following the in-frame stop codon at nucleotides 2-4. Preferably, the human HIF-1 $\alpha$  amino acid sequence is SEQ ID NO:2.

The nucleotide sequence encoding HIF-1 $\alpha$  includes SEQ ID NO:1 as well as nucleic acid sequences complementary to SEQ ID NO:1. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:2 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-identified nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA or RNA that encodes the polypeptide of SEQ ID NO:2 under physiological conditions. Specifically, the fragments should hybridize to DNA or RNA encoding HIF-1 $\alpha$  protein under stringent conditions.

Minor modifications of the HIF-1 $\alpha$  primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the HIF-1 $\alpha$  polypeptide described herein. Such proteins include those as defined by the term "having essentially the amino acid sequence of SEQ ID NO:2". Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of HIF-1 $\alpha$  still exists. Further, deletions of one or more amino acids can also result in modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which are not required for HIF-1 $\alpha$  biological activity.

The HIF-1 $\alpha$  polypeptide of the invention encoded by the nucleotide sequence of the invention includes the disclosed sequence (SEQ ID NO:2) and conservative variations thereof. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, or methionine for another, or the



substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that

5 antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

The DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization techniques which are well known in the art. These include, but are not limited to: 1) hybridization of

10 genomic or cDNA libraries with probes to detect homologous nucleotide sequences, 2) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to the DNA sequence of interest, and 3) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features.

15 Preferably the HIF-1 $\alpha$  nucleotide sequence of the invention is derived from a mammalian organism, and most preferably from human. Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the

20 protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequences must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a

25 heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by

30 using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Sambrook et al. (1989) Molecular

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Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY).

The development of specific DNA sequences encoding HIF-1 $\alpha$  can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that express the gene of interest at a high level. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay et al. (1983) Nucl. Acid Res., 11:2325).

A cDNA expression library, such as lambda gt11, can be screened indirectly for HIF-1 $\alpha$  peptides having at least one epitope, using antibodies specific for HIF-1 $\alpha$ . Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of HIF-1 $\alpha$  cDNA.

DNA sequences encoding HIF-1 $\alpha$  can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the HIF-1 $\alpha$  nucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the HIF-1 $\alpha$  genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription in the host of the inserted genetic sequence. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al. (1987) *Gene* 56:125), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans (1988) *J. Biol. Chem.* 263:3521) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedron promoters).

Nucleotide sequences encoding HIF-1 $\alpha$  can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and

subsequently treated by the  $\text{CaCl}_2$  method using procedures well known in the art. Alternatively,  $\text{MgCl}_2$  or  $\text{RbCl}$  can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

5 When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the HIF-1 $\alpha$  of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine  
10 kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see, for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

15 Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

The HIF-1 $\alpha$  polypeptides of the invention can also be used to produce antibodies which are immunoreactive or bind to epitopes of the HIF-1 $\alpha$   
20 polypeptides. Such antibodies can be used, for example, in standard affinity purification techniques to isolate HIF-1 $\alpha$  or HIF-1. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods  
25 well known in the art (Kohler et al. (1975) Nature 256:495; Current Protocols in Molecular Biology, Ausubel et al., ed., 1989).

For purposes of the invention, an antibody or nucleic acid probe specific for HIF-1 $\alpha$  may be used to detect HIF-1 $\alpha$  polypeptide (using antibody) or nucleotide sequences (using nucleic acid probe) in biological fluids or tissues. The antibody  
30 reactive with HIF-1 $\alpha$  or the nucleic acid probe is preferably labeled with a compound which allows detection of binding to HIF-1 $\alpha$ . Any specimen containing a detectable amount of antigen or polynucleotide can be used. Various detectable labels and assay formats are well known to those of ordinary skill in the art and can be utilized without resort to undue experimentation.

When the cell component is nucleic acid, it may be necessary to amplify the nucleic acid prior to binding with an HIF-1 $\alpha$  specific probe. Preferably, polymerase chain reaction (PCR) is used, however, other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The present invention provides a HIF-1 $\alpha$  variant polypeptide characterized as dimerizing with HIF-1 $\beta$  to form a functionally inactive HIF-1 complex in that the complex is not able to sufficiently bind to the HIF-1 binding motif in the regulatory region to allow efficient expression of the structural gene under control of the regulatory region. The invention further provides nucleotide sequences encoding HIF-1 $\alpha$  variants. In one specific embodiment, the polynucleotide encoding HIF-1 $\alpha$  variant is provided having the polynucleotide sequence of SEQ ID NO:3. The HIF-1 $\alpha$  variant polypeptide SEQ ID NO:4 is generated by substitution of wild-type amino acids with different amino acids and by deleting a portion of the wild-type sequence. Modifications of the HIF-1 $\alpha$  variant amino acid sequence are encompassed by the invention so long as the resulting polypeptide dimerizes to HIF-1 $\beta$  to form a functionally inactive HIF-1 complex in the sense that the HIF-1 complex or dimer no longer sufficiently binds DNA. In a preferred embodiment of the invention, specific HIF-1 $\alpha$  variants are provided wherein one or more the amino acids that participate in the binding of HIF-1 to DNA are replaced using techniques of genetic engineering.

The specific dominant-negative variant forms of HIF-1 $\alpha$  are HIF-1 $\alpha$  $\Delta$ NB and HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB (see Example 10). These two forms have in common a deletion of the amino acids that comprise the basic domain required for DNA binding (HIF-1 $\alpha$  amino acid residues 17-30; Fig. 10). Any variant form of HIF-1 $\alpha$  in which modification of the basic domain eliminates DNA binding activity while maintaining the ability of HIF-1 $\alpha$  to dimerize with HIF-1 $\beta$  should function as a dominant negative variant. Such alterations of the nucleotide sequence encoding the basic domain include deletions or substitutions of critical basic amino acid residues within the domain that are required for DNA binding. Additional modifications of the protein may enhance the dominant negative effect *in vivo*. For example, the HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB variant contains the same mutation in the basic domain as HIF-1 $\alpha$  $\Delta$ NB (Fig. 16) but, in addition, HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB is also truncated at the carboxy terminus to improve its protein stability *in vivo*.

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The nucleotide sequences encoding HIF-1 $\alpha$  variant molecules of the invention can be inserted into an appropriate expression vector and expressed in cells. Modified versions of the specific HIF-1 $\alpha$  variant of SEQ ID NO:4 can be engineered to enhance stability, production, purification, or yield of the expressed product. For example, the expression of a fusion protein or a cleavable fusion protein comprising the HIF-1 $\alpha$  variant and a heterologous protein can be engineered. Such a fusion protein can be readily isolated by affinity chromatography, e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the HIF-1 $\alpha$  moiety and the heterologous protein, the HIF-1 $\alpha$  polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that disrupts the cleavage site (Booth et al. (1988) *Immunol. Lett.* 19:65-708; Gardella et al. (1990) *J. Biol. Chem.* 265:15854-15859).

The invention provides methods for treatment of HIF-1-mediated disorders, including hypoxia-mediated tissue damage, which are improved or ameliorated by modulation of HIF-1 gene expression or activity. The term "modulate" envisions the inhibition of expression of HIF-1 when desirable, or enhancement of HIF-1 expression when appropriate. Where expression or enhancement of expression of HIF-1 is desirable, the method of the treatment includes direct (protein) or indirect (nucleotide) administration of HIF-1.

According to the method of the invention, substantially purified HIF-1 or the nucleotide sequence encoding HIF-1 is introduced into a human patient for the treatment or prevention of HIF-1-mediated disorders. The appropriate human patient is a subject suffering from a HIF-1-mediated disorder or a hypoxia-related disorder, such as atherosclerotic coronary or cerebral artery disease. When a patient is treated with nucleotide, the nucleotide can be a sequence which encodes HIF-1 $\alpha$  or a nucleotide sequence which encodes HIF-1 $\alpha$  and a nucleotide sequence which encodes HIF-1 $\beta$  (see, for example, Rayes, *et al.*, *Science*, 256:1193-1195, 1992; and Hoffman, *et al.*, *Science*, 252:954-958, 1991).

Where inhibition of HIF-1 $\alpha$  expression is desirable, such as the inhibition of tumor proliferation mediated by VEGF-induced angiogenesis, inhibitory nucleic acid sequences that interfere with HIF-1 expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes,

or triplex agents to block transcription or translation of a specific HIF-1 $\alpha$  mRNA or DNA, either by masking that mRNA with an antisense nucleic acid or DNA with a triplex agent, or by cleaving the nucleotide sequence with a ribozyme.

5 Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub (1990) Scientific American 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15  
10 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target HIF-1 $\alpha$ -producing cell.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix.  
15 Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher et al. (1991) Antisense Res. and Dev. 1:227; Helene (1991) Anticancer Drug Design, 6:569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA in a manner analogous to DNA restriction  
20 endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech (1988) J. Amer. Med. Assn. 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

25 There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff (1988) Nature 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11 - 18 bases in length. The longer the recognition sequence, the greater the likelihood that the  
30 sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

Suppression of HIF-1 function can also be achieved through administration of HIF-1 $\alpha$  variant polypeptide (dominant negative variant form), or a nucleotide sequence encoding HIF-1 $\alpha$  variant polypeptide. For example, in the case of disorders enhanced by expression of HIF-1 $\alpha$ , such as tumor proliferation  
5 secondary to VEGF-mediated angiogenesis, it would be desirable to "starve" the tumor by inhibiting neovascularization necessary to supply sufficient nutrients to the tumor. By administering HIF-1 $\alpha$  variant polypeptide or a nucleotide sequence encoding such polypeptide, the variant will compete with wild-type HIF-1 $\alpha$  for binding to HIF-1 $\beta$  in forming HIF-1 dimer thereby lowering the concentration of  
10 HIF-1 dimer in the cell which can efficiently bind to the HIF-1 DNA binding motif.

The present invention also provides gene therapy for the treatment of hypoxia-related disorders, which are improved or ameliorated by the HIF-1 polypeptide. Such therapy would achieve its therapeutic effect by introduction of the HIF-1 $\alpha$  nucleotide, alone or in combination with HIF-1 $\beta$  nucleotide, into cells  
15 exposed to hypoxic conditions. Delivery of HIF-1 $\alpha$  nucleotide, alone or in combination with HIF- $\beta$  nucleotide, can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Especially preferred for therapeutic delivery of sequences is the use of targeted liposomes.

20 Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, adeno-associated virus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine  
25 leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). Preferably, when the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a  
30 selectable marker so that transduced cells can be identified and generated. By inserting a HIF-1 $\alpha$  sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred



targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the HIF-1 $\alpha$  nucleotide sequence.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to  $\Psi$ 2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag*, *pol* and *env*, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for HIF-1 $\alpha$  nucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LW), which range in size from 0.2-4.0  $\mu$ m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al. (1981) Trends Biochem. Sci. 6:77). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene

transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al. (1988) Biotechniques 6:682).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with sterols, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidyl-glycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

Due to the biological activity of HIF-1 in enhancing synthesis of VEGF, EPO, and glycolytic enzymes, there are a variety of applications using the polypeptide or nucleotide of the invention. Such applications include treatment of hypoxia-related tissue damage and HIF-1-mediated disorders. In addition, HIF-1 may be  
5 useful in various gene therapy procedures. HIF-1 can be used to prevent or repair hypoxia-mediated tissue damage. Important applications include the treatment of cerebral and coronary artery disease.

Conversely, blocking HIF-1 action either with anti-HIF-1 antibodies, anti-HIF-1 $\alpha$  antibodies, or with an HIF-1 $\alpha$  antisense nucleotide might slow or ameliorate  
10 diseases dependent on HIF-1 action, e.g., V-EGF-promoted tumor vascularization. The above described method for delivering an HIF-1 $\alpha$  nucleotide are fully applicable to delivery of an HIF-1 antagonist for specific blocking of HIF-1 expression and/or activity when desirable. An HIF-1 antagonist can be an HIF-1 antibody, an HIF-1 $\alpha$  antibody, an HIF-1 $\alpha$  antisense nucleotide sequence, or the  
15 polypeptide or nucleotide of an HIF-1 $\alpha$  variant.

The isolation and purification of HIF-1 from EPO-producing Hep3B cells and non-EPO-producing HeLa S3 cells is described in Examples 1-3. HIF-1 protein was purified 11,250-fold by DEAE ion-exchange and DNA affinity  
20 chromatography. Analysis of HIF-1 revealed 4 polypeptides having molecular weights of 91, 93, 94 (HIF-1 $\beta$ ) and 120 kDa (HIF-1 $\alpha$ ). Glycerol gradient sedimentation analysis indicates that HIF-1 exists predominantly as a heterodimer and to a lesser extent as a heterotetramer.

The HIF-1 $\alpha$  polypeptide was isolated and sequenced. Its cDNA was generated by PCR and its sequence determined. The HIF-1 $\alpha$  polypeptide is  
25 characterized as a basic-helix-loop-helix (bHLH) polypeptide containing a PAS domain whose expression is regulated by cellular O<sub>2</sub> tension (Examples 4-7).

Induction of the transcription of genes encoding the glycolytic enzymes by HIF-1 was investigated (Example 9). The studies revealed that the glycolytic enzymes aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), and pyruvate  
30 kinase M (PKM) are induced by exposure of cells to HIF-1 inducers (1% O<sub>2</sub>, CoCl<sub>2</sub>, DFX). These genes have HIF-1 binding sites which were shown to specifically bind HIF-1. These results support the role of HIF-1 as a mediator of adaptive responses to hypoxia that underlie cellular and systemic oxygen homeostasis.

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A dominant-negative variant of HIF-1 $\alpha$  was generated lacking the basic domain (amino acid 17-30) of the protein which is required for the binding of HIF-1 to DNA (Example 10). The variant HIF-1 $\alpha$  subunit can dimerize with HIF-1 $\beta$ , but the resulting heterodimer cannot bind DNA. In cells overexpressing the variant  
5 HIF-1 $\alpha$  subunit, the majority of the HIF-1 $\beta$  subunits were engaged in non-functional heterodimers, resulting in functional inactivation of HIF-1. These results show that the HIF-1 $\alpha$  variant is useful *in vivo* for blocking HIF-1 activity.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to  
10 those skilled in the art may alternatively be used.

**Example 1.      Experimental Methods.**

Human HIF-1 was purified, and its DNA binding activity characterized as follows.

Cell Culture and Nuclear Extract Preparation.      Human Hep3B and HeLa  
15 cells were maintained and treated with 1% O<sub>2</sub> and CoCl<sub>2</sub> (Wang & Semenza (1993a) Proc. Natl. Acad. Sci. USA 90:4304-4308), and nuclear extracts were prepared as described previously (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454; Dignam et al. (1983) Nucleic Acids Res. 11:1474-1489). HeLa S3 cells, obtained from American Type Culture Collection were adapted to  
20 suspension growth in Spinner's minimum essential medium supplemented with 5% (v/v) horse serum (Quality Biological, Gaithersburg, MD). The cells were grown to a density of 8 x 10<sup>5</sup> cells/ml and maintained by dilution to 2 x 10<sup>5</sup> cells/ml with fresh complete medium every 2 days. For induction of HIF-1 DNA binding activity, HeLa S3 cells were treated with 125  $\mu$ M CoCl<sub>2</sub> for 4 h at 37 °C before  
25 harvesting by centrifugation for 10 min at 2,500 x g. Cell pellets were washed twice with ice cold phosphate-buffered saline and resuspended in 5 packed cell volumes of buffer A (10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl) supplemented with 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride and 1 mM Na<sub>3</sub>VO<sub>4</sub>. After incubation on ice for 10 min, cells were pelleted  
30 at 2,500 x g for 5 min, resuspended in 2 packed cell volumes of buffer A, and lysed by 20 strokes in a glass Dounce homogenizer with type B pestle. Nuclei were pelleted at 10,000 x g for 10 min and resuspended in 3.5 packed nuclear

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volumes of buffer C (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM  $\text{MgCl}_2$ ) supplemented with 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mM  $\text{Na}_3\text{VO}_4$ . Nuclear proteins were extracted by stirring at 4°C for 30 min. After centrifugation at 15,000 x g for 30 min, the supernatant was dialyzed against  
5 buffer Z-100 (25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , and 100 mM KCl) at 4°C. The dialysate was clarified by ultracentrifugation at 100,000 x g for 60 min at 4°C, and designated as crude nuclear extract. The nuclear extracts were aliquoted, frozen in liquid  $\text{N}_2$ , and stored at -80°C. Protein concentration was determined by  
10 the method of Bradford (1976) Anal. Biochem. 72:248-254, with a commercial kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Gel shift assays. Gel shift assays were performed as described (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454, herein specifically incorporated by reference) except that the binding reaction was in buffer Z-100. For gel shift  
15 assays with partially purified and affinity-purified HIF-1 preparations, 0.25 mg/ml of BSA and 0.05% Nonidet P-40 were included in the binding reaction. Nonspecific competitor calf thymus DNA (Sigma) was used in reduced amounts for partially purified fractions, and no calf thymus DNA was used for affinity-purified HIF-1 fractions. For competition experiments, unlabeled oligonucleotide  
20 DNA was incubated with DEAE-Sepharose column fractions for 5 min on ice before probe DNA was added.

Nuclear extracts prepared from HeLa cells cultured in the presence of 0, 5, 10, 25, 50, 75, 100, 250, 500 or 1000  $\mu\text{M}$   $\text{CoCl}_2$  for 4 h at 37°C, were incubated with W18 probe.

25 Methylation interference analysis. Methylation interference analysis was performed as described (Wang & Semenza (1993b) J. Biol. Chem. 268:21513-21518, herein specifically incorporated by reference), except 100  $\mu\text{g}$  of nuclear extract prepared from  $\text{CoCl}_2$ -treated HeLa cells were used in the binding reactions.

30 Results. To determine the optimal concentration of  $\text{CoCl}_2$  for induction of HIF-1 DNA binding activity, HeLa cells were treated with  $\text{CoCl}_2$ . Nuclear extracts were prepared and analyzed by gel shift assay with the wild-type oligonucleotide W18 (Example 2) as probe. Results are shown in Fig. 1. Induction of HIF-1 DNA binding activity by  $\text{CoCl}_2$  was dose-dependent. HIF-1 activity in nuclear extracts

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was detected at 25  $\mu\text{M}$   $\text{CoCl}_2$  and reached a peak activity at 250  $\mu\text{M}$ . Significant cell death, however, was observed at  $\text{CoCl}_2$  concentrations greater than 250  $\mu\text{M}$ , resulting in decreased yield of nuclear proteins. For this reason 125  $\mu\text{M}$   $\text{CoCl}_2$  was chosen for subsequent large scale nuclear extract preparation. Constitutive DNA binding activities, which also bind W18 probe sequence specifically remained relatively unchanged in cells treated with 0-100  $\mu\text{M}$   $\text{CoCl}_2$ , and decreased at  $\text{CoCl}_2$  concentration greater than 250  $\mu\text{M}$ , suggesting an adverse effect of high  $\text{CoCl}_2$  concentration on the cells. Nonspecific DNA binding activities were barely detectable in this particular gel shift assay and vary with cell type and the relative amount of nonspecific competitor DNA used.

Methylation interference analysis was performed to determine if HIF-1 from hypoxic Hep3B cells and  $\text{CoCl}_2$ -treated HeLa cells has the same DNA binding properties. As shown in Fig. 2, methylation of  $G_8$  or  $G_{10}$  on the coding strand eliminated or greatly reduced HIF-1 binding, respectively (Fig. 2, left, lane 2). Methylation of  $G_{10}$  only partially interfered with the binding of constitutive factors (Fig. 2, left, lanes 3 and 4). On the noncoding strand, methylation of  $G_7$  or  $G_{11}$  blocked HIF-1 binding to the probe (Fig. 2B, right, lane 2). Only the methylation of  $G_7$  interfered with binding of constitutive factors (Fig. 2B, right, lanes 3 and 4). The nonspecific binding activity was unaffected by DNA methylation on either strand (Fig. 2A, left, lane 5 and Fig. 2B, right, lane 5). The results indicate that (i) HIF-1 closely contacts  $G_8$  and  $G_{10}$  on the coding strand and  $G_7$  and  $G_{11}$  on the noncoding strand through the major groove of the DNA helix, and (ii) HIF-1 and the constitutive DNA binding factors can be distinguished by the nature of their DNA binding site contacts.

**Example 2.      Biochemical Purification of HIF-1.**

Preparation of DNA affinity columns. DNA affinity columns were prepared by coupling multimerized double-stranded oligonucleotides to CNBr-activated Sepharose (Kadonaga & Tijan (1986) Proc. Natl. Acad. Sci. USA 83:5889-5893).

5    The wild-type and the mutant column contained multimerized oligonucleotide W18 (SEQ ID NO:5) and M18 (SEQ ID NO:6) (mutation underlined), respectively.

W18:      5'-gatcGCCCTACGTGCTGTCTCA-3'  
          3'-CGGGATGCACGACAGAGTctag-5'

10    M18:      5'-gatcGCCCTAAAAGCTGTCTCA-3'  
          3'-CGGGATTTTCGACAGAGTctag-5'

Equal amounts of complementary oligonucleotides were annealed, phosphorylated, and ligated. Ligated oligonucleotides (60-500 bp) were extracted with phenol/chloroform, ethanol precipitated, resuspended in deionized water, and  
15    coupled to CNBr-activated Sepharose 4B as instructed by the manufacturer (Pharmacia Biotech Inc.). Approximately 50 ug of ligated double-stranded oligonucleotides were coupled per ml of Sepharose.

Purification of HIF-1. Crude nuclear extracts from 120 liters of CoCl<sub>2</sub>-treated HeLa S3 cells (435 ml, 3,040 mg) were thawed on ice and clarified by  
20    centrifugation at 15,000 x g for 10 min. Extracts were fractionated as three batches over a 36 ml DEAE-Sepharose CL-6B column (Pharmacia) in buffer Z-100 with a step gradient of increasing KCl. Fractions containing peak activity were pooled and dialyzed against buffer Z-100. The dialysate from DEAE-Sepharose columns was incubated with calf thymus DNA (Sigma) at a  
25    concentration of 4.4 ug/ml for 15 min on ice. After centrifugation at 15,000 x g for 10 min, the supernatant (240 ml; 2.3 mg/ml) was applied to a 6 ml DNA affinity column prepared with concatenated W18 oligonucleotide. The fractions containing HIF-1 activity were pooled and dialyzed against buffer Z-100. The dialysate from the first DNA-affinity column was mixed with calf thymus DNA at a  
30    concentration of 2.5 ug/ml and incubated on ice for 15 min. After centrifugation (as described above), the supernatant was applied to a 1.5 ml M18 DNA-Sepharose column. The flowthrough from the M18 column was collected and

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reapplied to a second 2 ml W18 column. All buffers used for DNA affinity chromatography were supplemented with 0.05% Nonidet P-40 and 5 mM DTT. The amount of protein in affinity column fractions was quantitated by silver staining of SDS-polyacrylamide gels or by Amido Black (Sigma) staining of nitrocellulose membranes (Schleicher & Schuell) spotted with protein samples and compared against known amounts of proteins standards (Bio-Rad).

For purification of HIF-1 from hypoxia-treated Hep3B cells, nuclear extracts (95 mg) were fractionated by the use of a 4 ml DEAE-Sepharose CL-6B column as described above. 0.25 M KCl elute fractions were dialyzed against buffer Z-100 and applied onto a Sephacryl S-300 gel filtration column (50 ml, 1.5 x 30 cm). The fractions containing HIF-1 activity were pooled and applied to a 2 ml calf thymus DNA column (0.8 mg of calf thymus DNA/ml of Sepharose) prepared by coupling single-stranded calf thymus DNA to CNBr-activated Sepharose 4B. The flowthrough was collected and applied to a 0.4 ml W18 column as described above after incubation with calf thymus DNA (2.2 ug/ml) for 10 min followed by another 0.2 ml W18 column after dialysis against buffer Z-100.

SDS-PAGE and Silver Staining. SDS-PAGE was carried out as described by Laemmli (1970) Nature 227:680-685. The gels were calibrated with high range molecular weight standards or prestained molecular weight markers (Bio-Rad). Electrophoresis was performed at 30 mA. Silver staining was performed with silver nitrate as described (Switzer et al. (1979) Anal. Biochem. 98:231-237). Molecular weight estimation for HIF-1 polypeptides was based on SDS-polyacrylamide gels with 3.2% cross-linking (acrylamide/bisacrylamide ratio of 30:1).

Results. Since HIF-1 DNA binding activity from hypoxic Hep3B cells and CoCl<sub>2</sub>-treated HeLa cells are indistinguishable (Example 1), HeLa S3 cells treated with 125 uM CoCl<sub>2</sub> were used as starting material for the large scale purification of HIF-1. To purify HIF-1 by DNA affinity chromatography, the constitutive DNA binding activity had to first be separated from HIF-1 since both bind specifically to the W18 DNA sequence. Various ion-exchange resins and gel filtration matrices were examined. HIF-1 was retained on DEAE anion-change resins in buffer Z-100, whereas constitutive DNA binding activity was found in the flowthrough. HIF-1 DNA binding activity was eluted with 250 mM KCl in buffer Z. DEAE-Sepharose chromatography effectively removed constitutive DNA binding



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activity and resulted in a 4-fold purification of HIF-1 (Fig. 3A, lanes 1 and 2). This step, however, appeared to destabilize the HIF-1 protein complex and resulted in a faster migrating form of HIF-1 (Fig. 3A, lane 2, second arrow), which was also occasionally seen in crude nuclear extract preparations. This faster migrating form could be converted to the slower migrating HIF-1 band at higher salt concentrations, and HIF-1 appeared predominantly as the slower migrating form again after the first round of DNA affinity column chromatography (Fig. 3A, lanes 10-12), suggesting that no HIF-1 component was lost during the DEAE-Sephacel chromatography step. Probe binding of both HIF-1 forms could be competed by unlabeled W18 (Fig. 3B, lanes 2-4) but not M18 oligonucleotide (Fig. 3B, lanes 5-7), which contained a three-base pair substitution that abolished the ability of the EPO enhancer to mediate hypoxia-inducible transcription.

Partially purified HIF-1 fractions were then incubated with nonspecific competitor calf thymus DNA at concentrations that allowed optimal detection of HIF-1 DNA binding activity by gel shift assays and applied to a W18 DNA affinity column. Eluted fractions containing HIF-1 (0.5 M KCl, Fig. 3A, lane 10; 1 M KCl, Fig. 3A, lane 11) were pooled and dialyzed against buffer Z-100. To eliminate nonspecific DNA-binding proteins that were not removed by calf thymus DNA competitor, the dialysate was applied to an M18 DNA column. HIF-1 DNA binding activity was detected in the flowthrough, which was then applied directly onto second W18 column. HIF-1 activity was detected exclusively in 0.5 M KCl fractions. Two rounds of W18 and one round of M18 column chromatography resulted in a purification of approximately 2,800-fold.

The results of the final large scale purification are summarized in Table 1. From 120 liters of HeLa cells, approximately 60  $\mu$ g of highly purified HIF-1 were obtained. The total purification was 11,250-fold and yielded approximately 22% of the starting of HIF-1 DNA binding activity. Our objective was to identify HIF-1 subunits and isolate HIF-1 components for the purpose of peptide mapping and protein microsequencing analysis. Since additional steps of purification resulted in markedly lower yield, we did not purify HIF-1 further to homogeneity. Aliquots from flowthrough of the M18 column (Fig. 4A, Load) as well as the 0.25 M KCl wash and 0.5 M KCl elute fractions of the second W18 column were analyzed by 6% SDS-PAGE and silver staining. Four polypeptides of 90-120 kDa were highly enriched in the 0.5 M KCl fraction, which had high HIF-1 DNA binding activity

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compared with the 0.25 M KCl fraction, which had very little HIF-1 activity. The 0.5 M KCl fraction, however, still had many of the contaminant proteins found in the 0.25 M KCl fraction.

In an initial pilot purification of HIF-1 from hypoxia-induced Hep3B cells, a different purification protocol was used. Gel filtration over a Sephacryl S-300 column was also found to be effective in separating HIF-1 from constitutive DNA binding activity. In addition, a calf thymus DNA column was used to remove nonspecific DNA-binding proteins prior to two rounds of W18 DNA affinity chromatography. HIF-1 activity was detected in 0.5 M KCl fractions from both DNA affinity columns. An aliquot from the 0.5 M KCl elute fraction of the first W18 column (Fig. 4B, Load) as well as the 0.25 M KCl wash and 0.5 M KCl elute fractions of the second W18 column were analyzed by 7% SDS-PAGE and silver staining. Four polypeptides of similar molecular mass to those that co-purified with HIF-1 DNA binding activity in  $\text{CoCl}_2$ -treated HeLa cells were present in the affinity-purified preparation from hypoxic Hep3B cells (Fig. 4B, lane 3, arrows), indicating that HIF-1 from the two different cell types is composed of the same polypeptide subunits. Affinity-purified HIF-1 from both  $\text{CoCl}_2$ -treated HeLa cells and hypoxic Hep3B cells bound specifically to the W18 probe in gel shift assays.

**Example 3.      Analysis of HIF-1 Subunits.**

The following experiments were conducted to identify polypeptides that are part of the HIF-1 DNA binding complex.

Preparative gel shift assays were performed with 30  $\mu\text{l}$  of affinity-purified HIF-1 and probe W18. Gel slices containing HIF-1 and surrounding areas were isolated after autoradiography with wet gel. Gel slices were placed on the stacking gel of a 6% SDS-polyacrylamide gel and incubated with Laemmli buffer *in situ* for 15 min, and electrophoresis was performed in parallel with 30  $\mu\text{l}$  of affinity-purified HIF-1 and molecular weight markers. For two-dimensional denaturing gel electrophoresis, two aliquots of affinity-purified HIF-1 were resolved on a 6% SDS-polyacrylamide gel with 5% cross-linking (acrylamide/bisacrylamide ratio of 19:1). One lane was stained with silver nitrate. The gel slices corresponding to regions of interest were isolated from the unstained lane. The isolated gel slices were placed directly on the stacking gel of the second dimension 6% SDS-polyacrylamide gel with 3.2% cross-linking, and electrophoresis was performed in parallel with 30  $\mu\text{l}$  of affinity purified HIF-1.

Peptide Mapping of HIF-1 Subunits. 2 ml of the affinity-purified HIF-1 were dialyzed against 10 mM ammonium bicarbonate, 0.05% SDS and lyophilized. After resuspension in a solubilizing solution (100 mM sucrose, 3% SDS, 21.25 mM Tris-HCl (pH 6.9), 1 mM EDTA, 5%  $\beta$ -mercaptoethanol, 0.005% bromphenol blue), the protein samples were heated to 37°C for 15 min and resolved on a 6% polyacrylamide gel containing 0.2% SDS. Polypeptides were transferred electrophoretically at 4°C to a polyvinylidene difluoride membrane (Bio-Rad) in 0.5 x Towbin buffer (Towbin et al. 1979) Proc. Natl. Acad. Sci. USA 76:4350-5354) (96 mM glycine, 12.5 mM Tris-HCl (pH 8.3)) with 10% acetic acid, destained with 5% acetic acid and rinsed with Milli-Q water. Membrane slices containing the HIF-1 polypeptides of 120, 94/93, and 91 kDa were excised and subjected to peptide mapping (Best et al. (1994) in Techniques in Protein Chemistry V (Crabb, J.W., ed.), pp. 205-213, Academic Press, San Diego, CA). *In situ* tryptic digestion and reverse phase HPLC were performed by the Wistar Protein Microchemistry Laboratory.

UV Cross-Linking Analysis. UV cross-linking was carried out as described (Wang & Semenza (1993) Proc. Natl. Acad. Sci. USA 90:4304-4308) except that 30  $\mu$ l of affinity-purified HIF-1 were used in the binding reaction. Affinity-purified HIF-1 was incubated with W18 probe in the absence or presence of unlabeled W18 or M18 oligonucleotide. After incubation for 15 min at 4°C, the reaction mixtures were irradiated with UV light (312 nm; Fisher Scientific) for 30 min and resolved by 6% SDS-PAGE with pre-stained molecular weight markers and visualized by autoradiography.

Glycerol Gradient Sedimentation. Linear gradients of 12 ml, 10-30% glycerol in a buffer containing 100 mM KCl, 25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 5 mM DTT, and 0.4 mM phenylmethylsulfonyl fluoride, were prepared for centrifugation in a Beckman SW40 rotor for 48 h at 4°C. Nuclear extract prepared from hypoxic Hep3B cells (100  $\mu$ l, 5 mg/ml) was mixed with an equal volume of glycerol gradient buffer containing 10% glycerol and layered on the top of the gradient. A marker gradient was sedimented in parallel and contained 50  $\mu$ g each of thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa) (Pharmacia). Markers were adjusted to the same volume and glycerol concentration as the sample. Fractions (0.5 ml)

were collected from the top of the tubes, and DNA binding activity was measured by the gel shift assay. Markers were assayed by SDS-PAGE and silver staining.

Results. In order to identify polypeptides that are part of the HIF-1 DNA binding complex, preparative gel shift assays were performed with affinity-purified HIF-1 and W18 probe. Gel slices containing the HIF-1-DNA complex were isolated, inserted directly into the wells of an SDS-polyacrylamide gel, and analyzed by electrophoresis in parallel with an aliquot of affinity-purified HIF-1 (Fig. 5A). Four polypeptides present in the HIF-1 complex migrated with an apparent molecular weight of 120, 94, 93, and 91 kDa, respectively (Fig. 5A, HIF-1). None of these peptides were detected in gel slices isolated from other regions of the same lane. These four polypeptides migrated at the same positions as the polypeptides that co-purified with HIF-1 DNA binding activity by DNA affinity chromatography (Fig. 5A, lane A). The 120 kDa polypeptide and the 91-94 kDa polypeptides appear to be present in an equimolar ratio, suggesting that the 120 kDa polypeptide forms complexes with any one of the 91-, 93-, and 94 kDa polypeptides.

On a 6% SDS-polyacrylamide gel with 3.2% cross-linking, the 120 kDa HIF-1 polypeptide migrated very close to a contaminant polypeptide of slightly greater apparent molecular weight (Fig. 5A, lane A), making isolation of the 120 kDa polypeptide difficult. This problem was resolved by separating the HIF-1 polypeptides on a 6% SDS-polyacrylamide gel with 5% cross-linking. The 120 kDa polypeptide migrated much faster on the more highly cross-linked gel relative to the migration of the 116 kDa molecular mass marker, whereas migration of the contaminant band (\*1) was unchanged (Fig. 5B, lane A). Under these conditions, however, the 91 kDa polypeptide ran very close to another contaminant band (\*2) below it. Two polyacrylamide gel systems with different degrees of crosslinking were therefore required for the isolation of the 91-94 kDa and the 120 kDa HIF-1 polypeptides, respectively.

To confirm that the HIF-1 polypeptides identified by the two gel systems were identical, two dimensional denaturing gel electrophoresis was performed. Affinity-purified HIF-1 was first resolved on a 6% SDS-polyacrylamide gel with 5% crosslinking (as in Fig. 5B, lane A). Regions of the gel containing the 120 kDa, 94/93/91-kDa HIF-1 polypeptides, as well as the two contaminant bands, were isolated and analyzed by electrophoresis on a 6% SDS-polyacrylamide gel with

3.2% crosslinking in parallel with an aliquot of the affinity-purified HIF-1. As shown in Fig. 5C, the isolated HIF-1 and contaminant polypeptides co-migrate with the corresponding bands in the control sample, indicating that the differences in their migration were due to different degrees of cross-linking of the SDS-polyacrylamide gels.

To determine whether the four polypeptides from the HIF-1 complex represent distinct protein species, tryptic peptide mapping was performed. The 91 kDa band was isolated individually while the 93 and 94 kDa bands were excised together after electrophoretic separation and transfer to a polyvinylidene difluoride membrane. Proteins were digested with trypsin *in situ*, and the tryptic peptides were separated by reverse phase HPLC (Fig. 6). The elution profiles of tryptic peptides derived from 91 kDa protein and 93/94 kDa proteins were nearly superimposable (Fig. 6), suggesting that they were derived from similar polypeptides. Another aliquot of HIF-1 was resolved on a 6% polyacrylamide gel of 5% crosslinking for isolation of the 120 kDa HIF-1 polypeptide. The tryptic peptide elution profile derived from the 120 kDa polypeptide was distinct from those of the 91-94 kDa polypeptides. These results suggest that HIF-1 is composed of two different subunits, 120 kDa HIF-1 $\alpha$  and 91/93/94 kDa HIF-1 $\beta$ .

To identify the DNA-binding subunit(s), affinity-purified HIF-1 was incubated with W18 probe. After UV irradiation to cross-link the DNA-binding proteins to nucleotide residues at the binding site, the reaction mixtures were boiled in Laemmli buffer and resolved by SDS-PAGE, and cross-linked proteins were visualized by autoradiography. Two DNA-binding proteins were detected (Fig. 7, lane 1). Their molecular masses were estimated to be approximately 120 and 92 kDa (after the 16 kDa molecular mass contributed by probe DNA was subtracted), similar to those of HIF-1 $\alpha$  and HIF-1 $\beta$ . The binding of both proteins to the probe was sequence-specific since it could be competed by unlabeled wild-type W18 (Fig. 7, lane 2) but not mutant M18 (Fig. 7, lane 3) oligonucleotide. These results suggest that both HIF-1 $\alpha$  and HIF-1 $\beta$  contact DNA directly. HIF-1 $\alpha$  was cross-linked to DNA much more strongly than HIF-1 $\beta$  (fig. 7, lanes 1 and 3). These data provided further evidence that the four polypeptides purified by DNA affinity chromatography are bona fide components of HIF-1 DNA binding activity.

To estimate the native size of HIF-1, glycerol gradient sedimentation analysis was performed with crude nuclear extract prepared from hypoxic Hep3B cells.

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HIF-1 and the constitutive DNA binding activity were monitored by gel shift assays. In hypoxic Hep3B nuclear extracts, HIF-I-DNA complexes are present in two forms, whereas in  $\text{CoCl}_2$ -treated HeLa extracts, the faster migrating form predominates. The results, shown in Fig. 8, demonstrate that the two bands of the HIF-1 doublet are separable by sedimentation. The faster migrating form was estimated to have a molecular mass of approximately 200-220 kDa. Longer exposure of the autoradiograph revealed that the slower migrating band co-migrated with ferritin, which has a molecular mass of 440 kDa. Assuming a globular conformation for both protein complexes, these results are consistent with the hypothesis that the faster migrating form represents a heterodimeric complex, consisting of a 120 kDa HIF-1 $\alpha$  subunit and a 91-94 kDa HIF-1 $\beta$  subunit, whereas the slower migrating form may represent a heterotetramer. The exact nature and stoichiometry of these HIF-I complexes, however, remains to be determined. The constitutive DNA binding activity has a molecular mass less than the 67 kDa BSA protein. Since UV cross-linking analysis indicated that the constitutive factor has a DNA-binding subunit of approximately 40-50 kDa, it is most likely that the constitutive factor binds DNA as a monomer. Consistent with the results of glycerol gradient sedimentation analysis, HIF-I eluted from a Sephacryl S-300 gel filtration column before the constitutive binding activity, and the slower migrating HIF-I gel shift activity eluted before the faster migrating form. These results suggest that HIF-I exists predominantly as a heterodimer in solution and to a lesser extent as a higher order complex, and that these complexes contain at least one HIF-1 $\alpha$  and one HIF-1 $\beta$  subunit.

**Example 4. Isolation and Characterization of HIF-1 $\alpha$  cDNA Sequences.**

**Protein microsequence analysis.** Purified HIF-I subunits were fractionated by SDS-polyacrylamide gel electrophoresis, and the 120 and 94 kDa polypeptides were transferred to polyvinylidene difluoride membranes, individually digested with trypsin *in situ* and peptides were fractionated by reverse-phase high-pressure liquid chromatography (Wang & Semenza (1995) J. Biol. Chem. 270:1230-1237, herein specifically incorporated by reference). Protein microsequence analysis was performed at the Wistar Protein Microchemistry Laboratory, Philadelphia (Best et al. (1994) *supra*).

**cDNA library construction and screening.** Poly (A)+ RNA was isolated from Hep3B cells cultured for 16 h at 37°C in a chamber flushed with 1%  $\text{O}_2$ /5%

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CO<sub>2</sub>/balance N<sub>2</sub>. cDNA was synthesized using oligo(dT) and random hexamer primers and bacteriophage libraries were constructed in  $\lambda$ gt11 and Uni-ZAP XR (Stratagene, La Jolla CA). cDNA libraries were screened with <sup>32</sup>P-labelled cDNA fragments by plaque hybridization as described (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY, herein specifically incorporated by reference).

PCR. Degenerate oligonucleotides primers were designed using codon preference rules (Lathe (1985) J. Mol. Biol. 183:1-12).  $\alpha$ F1 (5'-ATCGGATCCATCACIGA(A/G)CT(C/G)-ATGGGITATA-3') (SEQ ID NO:7) was based upon the amino terminus of HIF-1 $\alpha$  peptide 87-1 and used as a forward primer. Two nested reverse primers,  $\alpha$ R1 (5'-ATTAAGCmTGGT-(G/C)AGGTGGTCI(G/C)(A/T)GTC-3') (SEQ ID NO:8) and  $\alpha$ R2 (5'-ATTAAGCTTGCATGGTAGTA(T/C)TCATAGAT-3') (SEQ ID NO:9), were based upon the carboxy terminus of peptide 91-1. PCR was performed by: denaturation of 108 phage or 10 ng of phage DNA at 95°C for 10 min; addition of AmpliTaq (Perkin-Elmer) at 80°C; and amplification for 3 cycles at 95°C, 37°C, and 72°C (30 sec each) followed by 35 cycles at 95°C, 50°C, and 72°C (30 sec each). Nested PCR with  $\alpha$ F1/ $\alpha$ R1 and then  $\alpha$ F1/ $\alpha$ R2 generated an 86-bp fragment which was cloned into pGEM4 (Promega). For HIF-1 $\beta$  (ARNT), PCR was performed as described above using primers 5'-ATAAAGCTTGT(C/G)TA(C/T)GT-(C/G)TCIGA(C/T)TCIG-3' (SEQ ID NO:10) and 5'-ATCGAATTC(C/T)TCI-GACTGIGGCTGGTT-3' (SEQ ID NO:11) which resulted in the predicted 69-bp product. For analysis of the 5' end of HIF-1 $\beta$  (ARNT), Hep3B poly(A)+ RNA was reverse-transcribed using reagents from a 5'-RACE kit (Clontech). The cDNA was used as template to amplify nt 54-425 of ARNT cDNA (Hoffman et al. (1991) *supra*), with 5'-TACGGATCCGCCATGGCGGCGACT-ACTGA-3' (SEQ ID NO:12) (forward primer) and nested reverse primers 5'-AGCCAGGGCACTACAGGTGGGTACC-3' (SEQ ID NO:13) and 5'-GTTCCCCGCAAGGACTTCATGTGAG-3' (SEQ ID NO:14) for 35 cycles at 95°C, 60°C, and 72°C (30 sec each). PCR products were cloned into pGEM4 for nucleotide sequence analysis.

Results. The purified 120 kDa HIF-1 $\alpha$  polypeptide was digested with trypsin, peptides were fractionated by reverse-phase high-pressure liquid chromatography and fractions 87 and 92 were subjected to microsequencing. Each fraction

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contained two tryptic peptides, for which virtually complete amino acid sequences were obtained: ITELMGYPEELLGR (SEQ ID NO:15) (87-1), XIILIPSDLAXR (SEQ ID NO:16) (87-2), SIYEYYHALDSDHLTK (SEQ ID NO:17) (91-1), and SFFLR (SEQ ID NO:18) (91-2). When 87-1 and 91-1 were entered as contiguous sequences, database searches identified similarities to the *Drosophila* proteins period (PER) and single-minded (SIM), and the mammalian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins, which all contain sequences of 200-350 amino acids that constitute the PAS (PER-ARNT-AHR-SIM) domain (Hoffman et al. (1991) Science 252:954-958; Citri et al. (1987) Nature 326:42-47; Burbach et al. (1992) Proc. Natl. Acad. Sci. USA 89:8185-8189; Crews et al. (1988) Cell 52:143-151; Nambu et al. (1991) Cell 67:1157-1167). Degenerate oligonucleotides were synthesized based upon the 87-1 and 91-1 sequences and used for PCR with cDNA prepared from hypoxic Hep3B cells. Nucleotide sequence analysis revealed that the cloned PCR product encoded the predicted amino acids, demonstrating that 87-1 and 91-1 were contiguous peptides.

**Example 5.      Nucleotide sequence and database analysis.** Complete unambiguous double stranded nucleotide sequences were obtained by incorporation of fluorescence-labeled dideoxy nucleotides into thermal-cycle sequencing reactions using T3, T7, and custom-synthesized primers. Reactions were performed using Applied Biosystems 394 DNA Synthesizers and 373a Automated DNA Sequencers in the Genetics Core Resources Facility of The Johns Hopkins University. Protein and nucleic acid database searches were performed at the National Center for Biotechnology Information using the programs BLASTP and TBLASTN (Altschul et al. (1990) J. Mol. Biol. 215:403-410). The HIF-1 $\alpha$  cDNA nucleotide sequence and deduced amino acid sequence have been submitted to GenBank. The accession number is U22431.

**Results.** Database analysis also identified an expressed-sequence tag (EST) whose derived amino acid sequence showed similarity to bHLH-PAS proteins. We obtained the 3.6-kb cDNA from which the EST was derived, hbc025 (Takeda et al. (1993) Hum. Mol. Genet. 2:1793-1798). Complete nucleotide sequence analysis revealed that it encoded all four tryptic peptides. Another EST was identified which shared identity with hbc025 and was encoded by a 2.0-kb cDNA,



hbc120 (Takeda et al. (1993) supra). Sequence analysis of hbc120 revealed that it was co-linear with the 3' end of hbc025 (Fig. 9), differing only in the length of the poly (A) tail. The 5' end of hbc025 was used to screen a Hep3B cDNA library, resulting in the isolation of an overlapping 3.4-kb cDNA, 3.2-3, which extended to an initiator codon. The composite cDNA of 3720 bp encoded a 2478-bp open reading frame that included a translation initiation codon, a 28-bp 5'-untranslated region (5'-UTR) that contained an in-frame termination codon, and a 1211-bp 3'-UTR that ended with a canonical polyadenylation signal followed after 12 bp by 43 adenine residues. Compared to the consensus translation-initiation sequence GCC(A/G)CCATGG (SEQ ID NO:19) (Kozak (1987) Nucleic Acids Res. 15:8125-8132), the HIF-1 $\alpha$  cDNA sequence is TTCACCATGG (SEQ ID NO:20). The HIF-1 $\alpha$  cDNA open reading frame predicted a novel 826 amino acid polypeptide (Fig. 10) with a molecular mass of 93 kDa that contained a bHLH-PAS domain at its amino terminus.

Analysis of two tryptic peptides isolated from the 94 kDa HIF-1 $\beta$  polypeptide (Wang & Semenza (1995) supra) yielded partial amino acid sequences, VVYVSDSVTPVLNQPQSE (SEQ ID NO:21) and TSQFGVGSFQTPSSFSSMXLPGAPTASPGAAAY (SEQ ID NO:22). Using degenerate oligonucleotides based upon the second peptide sequence, a PCR product of the predicted size was amplified from Hep3B cDNA. Database searches identified both peptides within the sequence of ARNT, a bHLH-PAS protein previously shown to heterodimerize with AHR to form the functional dioxin receptor (Reyes et al. (1992) Science 256:1193-1195). Two isoforms of ARNT have been identified which differ by the presence or absence of a 15 amino acid sequence encoded by a 45-bp alternative exon (Hoffman et al. (1991) supra). Analysis of Hep3B RNA by reverse transcriptase-PCR revealed the presence of both sequences, as well as additional isoforms. These primary sequence differences may account for the purification of three (91,93, and 94 kDa) HIF-1 $\beta$  polypeptides (Wang & Semenza (1995) supra). The apparent molecular mass of both HIF-1 $\alpha$  and HIF-1 $\beta$  on denaturing gels was greater than the mass predicted from the cDNA sequence. For HIF-1 $\alpha$  the apparent mass was 120 kDa compared to a calculated mass of 93 kDa; for the HIF-1 $\beta$  subunits, the apparent masses were 91-94 kDa compared to calculated masses of 85 and 87 kDa for the 774 and 789 amino acid isoforms of ARNT, respectively. The HIF-1 $\alpha$  and ARNT

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sequences contain multiple consensus sites for protein phosphorylation and HIF-1 has been shown to require phosphorylation for DNA binding (Wang & Semenza (1993b) supra).

5 HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) belong to different classes of bHLH domains, which consist of contiguous DNA binding (b) and dimerization (HLH) motifs. The bHLH domain of HIF-1 $\alpha$  is most similar to the other bHLHPAS proteins, SIM and AHR (Fig. 11). HIF-1 $\beta$  (ARNT) has greatest similarity to the bHLH domains found in a series of mammalian (MI, USF, L-MYC) and yeast (CP- 1) proteins that bind to 5'-CACGTG-3' (SEQ ID NO:23) (Dang et al. (1992) Proc. Natl. Acad. Sci. USA 89:599-603), a sequence which resembles the HIF-1 [5'-(G/Y)ACGTGC(G/T)-3' (SEQ ID NO:24) (Semenza et al. (1994) supra)] and dioxin receptor [5'-(TIG)NGCGTG(A/C)-(G/C)A-3' (SEQ ID NO:25) (Lusska et al. (1993) J. Biol Chem. 268:6575-6580)] binding sites. These transcription factors share bHLH domains of related sequence which occur in different dimerization contexts: MI, 10 L-MYC, and USF are bHLH-leucine zipper proteins, ARNT is a bHLH-PAS protein, and CP-1 contains only a bHLH domain.

Analysis of PAS domains, which have been implicated in both ligand binding and protein dimerization (Huang et al. (1993) Nature 364:259-262; Dolwick et al. (1993) Proc. Natl. Acad. Sci. USA 90:8566-8570; Reisz-Porszasz et al. (1994) 20 Mol. Cell. Biol. 14:6075-6086), revealed that HIF-1 $\alpha$  is most similar to SIM. Our alignment established consensus sequences that include a previously unreported motif, HXXD, present in the A and B repeats of all PAS proteins (Fig. 12). We also found that KinA of *Bacillus subtilis* (Perego et al. (1989) J. Bacteriol. 171:6187-6196) contains a PAS domain at its amino terminus and is thus the first procaryotic member of this protein family, indicating a remarkable degree of evolutionary conservation. KinA, like PER, possesses a PAS but not a bHLH domain and is thus unlikely to bind DNA. *B. subtilis* undergoes sporulation in response to adverse environmental conditions and KinA functions as a sensor that transmits signals via a carboxy-terminal kinase domain (Burbulys et al. (1991) 25 Cell 64, 545-552).

#### Example 6. RNA Blot Hybridization.

The expression of HIF-1 RNAs in response to inducers of HIF-1 DNA-binding activity was analyzed as follows.

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Total RNA (15 ug) was fractionated by 2.2 M formaldehyde/ 1.4% agarose gel electrophoresis, transferred to nitrocellulose membranes and hybridized at 68°C in Quik-Hyb (Stratagene) to <sup>32</sup>P-labelled HIF-1α or ARNT cDNA. Gels were stained with ethidium bromide and RNA was visualized by ultraviolet illumination before and after transfer to insure equal loading and transfer, respectively, in each lane. Based upon the migration of RNA size markers (BRL-GIBCO) on the same gels, the size of HIF-1α RNA was estimated to be 3.7 ± 0.1 kb. Two ARNT RNA species were identified as previously reported (Hoffman et al. (1991) supra).

Results. When Hep3B cells were exposed to 1% O<sub>2</sub>, HIF-1α and HIF-1β (ARNT) RNA levels peaked at 1-2 h, declined to near basal levels at 8 h, and showed a secondary increase at 16 h of continuous hypoxia (Fig. 13A). In response to 75 uM CoCl<sub>2</sub>, HIF-1 RNAs peaked at 4 h, declined at 8 h, and increased again at 16 h (Fig. 13B). In cells treated with 130 uM desferrioxamine, a single peak at 1-2 h was seen (Fig. 13C). When cells were incubated at 1% O<sub>2</sub> for 4 h and then returned to 20% O<sub>2</sub>, both HIF-1α and HIF-1β RNA decreased to below basal levels within 5 min, the earliest time point assayed (Fig. 13D). These results demonstrate that, as in the case of HIF-1 DNA-binding activity (Wang & Semenza (1993b) supra), HIF-1 RNA levels are tightly regulated by cellular O<sub>2</sub> tension. The marked instability of HIF-1α RNA in posthypoxic cells may involve the 3'-untranslated region (3'-UTR) which contains eight AUUUA sequences (Fig. 13E) that have been identified in RNAs with short half-lives and shown to have a destabilizing effect when introduced into heterologous RNAs (Shaw & Kamen (1986) Cell 46:659-667). Seven of the HIF-1α AUUUA sequences conform to a more stringent consensus for RNA instability elements, 5'-UUAUUUA(U/A)(U/A)-3' (SEQ ID NO:26) (Lagnado et al. (1994) Mol. Cell. Biol. 14:7984-7995).

#### **Example 7.      Antibody Production.**

To analyze HIF-1 protein expression, polyclonal antisera was raised against HIF-1α and HIF-1β as follows.

Rabbits were immunized with recombinant proteins in which glutathione-S-transferase (GST) was fused to amino acids 329-531 of HIF-1α or 496-789 of ARNT. To generate antibodies against HIF-1α, a 0.6 kb EcoRI fragment from hbc025 was cloned into pGEX-3X (Pharmacia) and transformed

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into *E. coli* DH5 $\alpha$  cells (GIBCO-BRL). GST/HIF-1 $\alpha$  fusion protein was isolated by exposure of bacteria (OD<sub>600</sub> = 0.8) to 0.1 mM IPTG at room temperature for 1 h; sonication in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride; centrifugation at 10,000 x g for 10 min; incubation of supernatant with glutathione-agarose (Pharmacia) in the presence of 1% NP-40 for 1 h at 4°C; and elution with 5 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl. To generate antibodies against HIF-1 $\beta$ , ARNT nt 1542-2428 were amplified from Hep3B cDNA by PCR with Taq polymerase using forward primer 5'-ATAGGATCCTCAGGTCAGCTGGCACCCAG-3' (SEQ ID NO:27) and reverse primer 5'-CCAAAGCTTCTATTCTGAAAAGGGGGG-3' (SEQ ID NO:28). The product was digested with BamHI and EcoRI, to generate a fragment corresponding to ARNT nt 1542-2387, and cloned into pGEX-2T (Pharmacia). Fusion protein isolation was as described above, except that induction was with 1 mM IPTG for 2 h and binding to glutathione-agarose was in the presence of 1% Triton X-100 rather than NP-40. Fusion proteins were excised from 10% SDS/polyacrylamide gels and used to immunize New Zealand white rabbits (HRP Inc., Denver PA) according to an institutionally-approved protocol. Antibodies raised against HIF-1 $\alpha$  were affinity-purified by binding to GST/HIF-1 $\alpha$  coupled to CNBr-activated Sepharose 4B (Pharmacia).

**Results.** Antisera was used to demonstrate that the proteins encoded by the cloned HIF-1 $\alpha$  cDNA and ARNT are components of HIF-1 DNA-binding activity (Fig. 14A). When crude nuclear extracts from hypoxic cells were incubated with probe DNA and either antiserum, the HIF-1/DNA complex seen in the absence of antisera was replaced by a more slowly migrating HIF-1/DNA/antibody complex, whereas addition of preimmune sera had no effect on the HIF-1/DNA complex.

#### **Example 8.      Immunoblot analysis.**

15 ug aliquots of nuclear protein extracts were resolved on 6% SDS/polyacrylamide gels and transferred to nitrocellulose membranes in 20 mM Tris-HCl (pH 8.0), 150 mM glycine, 20% methanol. Membranes were blocked with 5% milk/TBS-T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20], incubated with affinity-purified HIF-1 $\alpha$  antibodies or HIF-1 $\beta$  antiserum diluted 1:400 or 1:5000, respectively, washed, incubated with horseradish peroxidase anti-immunoglobulin conjugate diluted 1:5000, washed, and developed with ECL

reagents (Amersham) and autoradiography. Incubations were for 1 h in 5% milk/TBS-T and washes were for a total of 30 min in TBS-T at room temperature.

Results. Immunoblot analysis revealed that the antisera detected polypeptides in crude nuclear extracts from hypoxic Hep3B or CoCl<sub>2</sub>-treated HeLa cells which co-migrated with polypeptides present in purified HIF-1 protein preparations (Fig. 14B). Analysis of nuclear and cytoplasmic extracts prepared from Hep3B cells exposed to 1% O<sub>2</sub> (Fig. 14C) revealed that peak levels of HIF-1 $\alpha$  and HIF-1 $\beta$  were present in nuclear extracts at 4-8 h of continuous hypoxia, similar to the induction kinetics of HIF-1 DNA-binding activity (Wang & Semenza (1993) J. Biol. Chem. 268:21513-21518). For HIF-1 $\alpha$ , the predominant protein species accumulating at later time points migrated to a higher position in the gel than protein present at earlier time points, suggesting that post-translational modification of HIF-1 $\alpha$  may occur. For HIF-1 $\beta$ , the 94- and 93 kDa species were resolved from the 91 kDa form but not from each other and no shifts in migration were seen. The post-hypoxic decay of HIF-1 proteins was also remarkably rapid (Fig. 14D), indicating that, as with the RNAs, these proteins are unstable in post-hypoxic cells. For both HIF-1 $\alpha$  and ARNT, 31% of all amino acids are proline, glutamic acid, serine, or threonine (PEST) residues, which have been implicated in protein instability (Rogers et al. (1986) Science 234:364-368). In HIF-1 $\alpha$ , two 20 amino acid sequences (499-518 and 581-600; Fig. 10) each contain 15 PEST residues. For HIF-1 $\beta$  (ARNT), redistribution between nuclear and cytoplasmic compartments also appeared to play a role in both the induction and decay of nuclear protein levels.

Together with our previous studies of HIF-1, the results presented here indicate that HIF-1 is a heterodimeric bHLH-PAS transcription factor consisting of a 120 kDa HIF-1 $\alpha$  subunit complexed with a 91-94 kDa HIF-1 $\beta$  (ARNT) isoform. Thus, ARNT encodes a series of common subunits utilized by both HIF-1 and the dioxin receptor, analogous to the heterodimerization of E2A gene products with various bHLH proteins (Murre et al. (1989) Cell 58:537-544). Based upon these results and the similarity of HIF-1 $\alpha$  and SIM within the bHLH-PAS domain, ARNT may also heterodimerize with SIM. In *Drosophila*, several SIM-regulated genes are characterized by enhancer elements that include 1-5 copies of the sequence 5'-(G/A)(T/A)ACGTG-3' (SEQ ID NO:29)(Wharton et al. (1994) Development 120:3563-3569). The observation that the HIF-1, dioxin receptor, and SIM

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binding sites share the sequence 5'-CGTG-3' supports the hypothesis that ARNT is capable of combinatorial association with HIF-1 $\alpha$ , AHR, and SIM since this half-site is also recognized by the transcription factors with which ARNT shows greatest similarity in the bHLH domain.

5      **Example 9.      Transcriptional Regulation of Genes Encoding Glycolytic Enzymes by HIF-1.**

The involvement of HIF-1 in transcriptional regulation of genes encoding glycolytic enzymes in hypoxic cells was investigated as follows.

10      RNA analysis. Total RNA was isolated from Hep3B and HeLa cells (Chomczynski & Sacchi (1987) Anal. Biochem 162:156-159). RNA concentrations were determined by absorbance at 260 nm. Agarose gel electrophoresis, followed by ethidium bromide staining and visualization of 28 and 18 S rRNA under UV illumination, confirmed that aliquots from different preparations contained equal amounts of intact total RNA. Plasmids N-KS<sup>+</sup> and 15      H-KS<sup>+</sup>, provided by P. Maire (Institut Cochin de Genetique Moleculaire, Paris), were linearized by digestion with HindIII. Antisense RNA was synthesized by T3 RNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]ATP. 10 ug of total cellular RNA was hybridized to H or N riboprobe (3 x 10<sup>5</sup> cpm) for 3 h at 66°C and digested with RNases A and T<sub>1</sub>; protected fragments 20      were analyzed by 8 M urea, 8% polyacrylamide gel electrophoresis (Semenza et al. (1990) Mol. Cell. Biol. 10:930-938). Human phosphoglycerate kinase 1 (PGKI) cDNA from plasmid pHPGK-7e (Michelson et al. (1985) Proc. Natl. Acad. Sci. USA 82:6965-6969), obtained from American Type Culture Collection, and rat PKM cDNA from plasmid pM2PK33 (Noguchi et al. (1986) J. Biol. Chem. 25      261:13807-13812), provided by T. Noguchi (Osaka University Medical School, Osaka, Japan), were used as random-labeled probes for blot hybridizations performed in QuikHyb (Stratagene) for 1 h at 68 °C, followed by washing in 15 mM sodium chloride, 1.5 mM sodium citrate, 0.1% SDS at 50 °C. Densitometric analysis of autoradiograms was performed with an LKM Ultrosan XL laser 30      densitometer using computerized peak integration.

Electrophoretic Mobility Shift Assay (EMSA). Crude nuclear extract preparations, conditions of probe preparation, binding reactions, and gel analysis were all previously described above. Double-stranded oligonucleotides were

synthesized according to the sequences shown in Table 2 except that each oligonucleotide contained at its 5'-end the sequence 5'-GATC-3', which formed a single-stranded 5' overhang when complementary oligonucleotides were annealed. The sense strand sequence of the W18 and M18 oligonucleotides was as given above. HIF-1 was partially purified from 50 liters of CoCl<sub>2</sub>-treated HeLa cells by crude nuclear extract preparation, DEAE-Sepharose chromatography, MonoQ fast protein liquid chromatography, and DNA affinity chromatography. Incubations with crude nuclear extracts and partially purified HIF-1 contained 100 and 1 ng of denatured calf thymus DNA, respectively. Competition experiments were performed with 5 ng of unlabeled W18 or M18 oligonucleotide.

Tissue culture. Hep3B and HeLa cells were maintained in culture and treated with 1% O<sub>2</sub>, CoCl<sub>2</sub>, DFX, and cycloheximide (CHX) as described above.

Transient Expression Assay. The psvcat reporter plasmid (pCAT Promoter, Promega) contained SV40 early region promoter, bacterial chloramphenicol acetyltransferase (CAT) coding sequences, SV40 splice, and polyadenylation signals. Oligonucleotides were cloned into the BglII and BamHI sites located 5' and 3' to the transcription unit, respectively. Plasmids pNMHcat and pHcat (Concordet et al. (1991) Nucleic Acids Res. 19:4173-4180), containing human aldolase A gene sequences fused directly to CAT coding sequences, were provided by P. Maire. pSVβgal (Promega) contained bacterial lacZ coding sequences driven by the SV40 early region promoter and enhancer. Plasmids were purified by alkaline lysis and two rounds of cesium chloride density gradient centrifugation. Hep3B cells were transfected by electroporation with a Gene Pulser (Bio-Rad) at 260 V and 960 microfarads. Duplicate electroporations were pooled and split onto two 10 cm tissue culture dishes (Corning) containing 8 ml of media. Cells were allowed to recover for 24 h in a 5% CO<sub>2</sub> 95% air incubator at 37°C, the media was replaced, and one set of duplicate plates was removed to a modular incubator chamber, which was flushed with 1% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>, sealed, and placed at 37°C. Cells were harvested 72 h after transfection, and extracts were prepared for CAT and β-galactosidase activity.

Results. The human aldolase A gene (hALDA) contains four noncoding exons, N1, N2, M, and H (Maire et al. (1987) J. Mol. Biol. 197:425-438). Transcription is initiated at exons N1 and H in most tissues other than muscle. Ribonuclease protection assays of RNA isolated from cells exposed to 20 or 1%

O<sub>2</sub> for 16 h revealed 3.0- and 2.9-fold higher levels of ALDA RNA initiated from exon H in Hep3B and HeLa cells exposed to 1% O<sub>2</sub>, whereas RNA initiated from exon N1 increased only 1.7- and 1.1-fold in hypoxic Hep3B and HeLa cells, respectively, suggesting a promoter-specific response to hypoxia.

5        We next compared the expression of ALDA and phosphoglycerate kinase 1 (PGKI) RNA in Hep3B cells exposed to 1% O<sub>2</sub> for 0-16 h. Maximal induction of both ALDA and PGK1 RNA showed delayed kinetics, suggesting a requirement for protein synthesis during induction, which was confirmed by the demonstration that treatment of Hep3B cells with 100  $\mu$ M CHX decreased induction of ALDA and  
10        PGK1 RNA in hypoxic cells from 6.1- and 8.2-fold to 1.6- and 1.4-fold, respectively.

Treatment of Hep3B cells for 16 h with 75  $\mu$ M CoCl<sub>2</sub> or 130  $\mu$ M DFX induced both ALDA and PGK1 RNA with ALDA transcripts preferentially initiated from exon H. Analysis of the same RNA samples with a probe for PKM revealed that  
15        PKM RNA was also induced by exposure of Hep3B cells to 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX. ALDA, PGK1, and PKM RNAs were also induced by treatment of HeLa cells with 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX. PFKL RNA was not expressed at detectable levels in Hep3B or HeLa cells. These RNA analyses demonstrate that agents that induce EPO RNA and HIF-1 activity also induce ALDA, PGK1, and PKM RNA in both  
20        EPO-producing Hep3B and nonproducing HeLa cells, with a requirement for *de novo* protein synthesis, as previously demonstrated for induction of EPO RNA and HIF-1 activity (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454).

Nucleotide sequences of genes encoding glycolytic enzymes present in Gen-Bank were searched for potential HIF-1 binding sites using the query sequence  
25        5'-ACGTGC-3', which contains the 4 guanine residues that contact HIF-1 in the DNA major groove (Wang & Semenza (1993b) *supra*). Double-stranded oligonucleotides were synthesized corresponding to 5'-flanking sequences (5'-FS) of the human PGK1 (hPGKI), human enolase 1 (hENO1), and mouse LDHA (mLDHA) genes; 5'-untranslated sequences (5'-UT) of hPGKI; and intervening  
30        sequences (IVS) of the hALDA and mPFKL genes. These oligonucleotides contained, as potential HIF-1 sites, 5'-TACGTGCT-3' (SEQ ID NO:30), 5'-GACGTGCG-3' (SEQ ID NO:31) (which was also found in hEPO 5'-FS), and 5'-CACGTGCG-3' (SEQ ID NO:32). The first sequence is identical to the previously identified HIF-1 binding site in the EPO enhancer (Semenza & Wang



(1992) supra), whereas the latter two sequences differ at the first and last nucleotides. The ability of these oligonucleotides to bind HIF-1 was tested by EMSA.

5 When incubated with nuclear extract prepared from Hep3B cells exposed to 1% O<sub>2</sub> for 4 h, each probe generated a DNA protein complex of similar mobility and intensity to the HIF-1 complex formed with probe W18, corresponding to nucleotides 1-18 of the hEPO 3'-FS. In contrast, none of these probes detected an HIF-1 complex in nuclear extracts from cells maintained at 20% O<sub>2</sub>, although the EMSA patterns were otherwise similar to those obtained with nuclear extracts from hypoxic cells. The DNA-protein complex migrating below the HIF-1 complex was less intense when hypoxic (compared with non-hypoxic) nuclear extracts were assayed. We have previously shown that this complex contains a  
10 constitutively expressed factor that recognizes the same DNA sequence as HIF-1 (Wang & Semenza (1993b) supra). The decreased binding of the constitutive factor may thus result from competition for binding with HIF-1 in hypoxic extracts.  
15

EMSA was also performed with a preparation of HIF-1 from CoCl<sub>2</sub>-treated HeLa cells that was purified approximately 600-fold by DEAE-cellulose, MonoQ, and DNA affinity chromatography. Each probe bound HIF-1 in a manner that was qualitatively and quantitatively similar to the complex formed with W18. The  
20 binding of HIF-1 to these probes was sequence-specific as it could be competed by an excess of unlabeled W18 but not by mutant oligonucleotide M18, containing a 3-nucleotide substitution previously shown to eliminate HIF-1 binding and hypoxia-inducible enhancer function. Similar results were obtained when competition experiments involving W18 and M18 were performed with crude  
25 nuclear extract from hypoxic Hep3B cells. These results identify novel HIF-1 binding sites in genes encoding ALDA, ENO1, PFKL, and PGK1 as well as in the hEPO 5'-FS. The 8 oligonucleotides that have been shown to specifically bind HIF-1 (Table 2) contain 3 different binding site sequences that are represented by the consensus 5'-(C/G/T)ACGTGC(G/T)-3' (SEQ ID NO:33). Given the biased  
30 method of ascertainment, it is possible that HIF-1 may recognize other sequences not represented by this consensus. In addition to the 6 HIF-1 sites from glycolytic genes, the sequence 5'-CACGTGCT-3' (SEQ ID NO:34) was also present in the hENO1 5'-FS at -786 to -793 (Gialongo et al. (1990) Eur. J. Biochem. 190:567-573) but was not tested for HIF-1 binding. Thus, a total of 7 probable HIF-1 sites

were identified in 20.7 kb of nucleotide sequence reported to GenBank for these 5 glycolytic genes. In contrast, no sequences matching the consensus HIF-1 site were identified on either DNA strand within a total of 43.5 kb, comprising the nucleotide sequences of 5 randomly chosen genes, AFP, BUP4, CREB, DHFR, and EPOR (Gibbs et al. (1987) *Biochemistry* 26:1332-1343; Kurihara et al. (1993) *Biochem. Biophys. Res. Commun.* 192:1049-1056; Meyer et al. (1993) *Endocrinology* 132:770-780; Mitchell et al. (1986) *Mol. Cell. Biol.* 6:425-440; Noguchi et al. (1991) *Blood* 78:2548-2556).

To determine whether these HIF-1 binding sites were of functional importance, transient expression essays were performed using the reporter genes described above. Reporter plasmids were cotransfected into Hep3B cells with pSV $\beta$ gal, which was included as a control for variation in transfection efficiency. Transfected cells were split among duplicate plates that were cultured in 1 or 20% O<sub>2</sub> for 48 h, CAT and  $\beta$ -galactosidase protein synthesized following transcription of reporter and control plasmids, respectively, were quantitated from cellular extracts. The basal reporter psvcat, in which transcription of CAT coding sequences was driven by the SV40 early region promoter, generated similar CAT/ $\beta$ -galactosidase values in cells cultured at 1 and 20% O<sub>2</sub>. When one (psvcatEPO1) or two (psvcatEPO2) copies of the 33-base pair hEPO 3'-FS enhancer were cloned 3' to the transcription unit, CAT/ $\beta$ -galactosidase expression was induced 4.9- and 17-fold, respectively, in cells cultured at 1% O<sub>2</sub>, consistent with previously reported results (Semenza & Wang (1992) *supra*).

HIF-1 binding site sequences from glycolytic genes were analyzed in the same assay. The mPFKL IVS-1 and hPFK1 5'-FS oligonucleotides were chosen, as they represented sequences identical to or divergent from the HIF-1 site in the hEPO 3'-FS and were located 3' or 5' to the transcription initiation site, respectively. Two copies of the 24-base pair hPGK1 5'-FS oligonucleotide were cloned 5' to the psvcat transcription unit (Fig. 15A), analogous to its location in hPGK1. Expression of pPGK2svcat was induced 5.6-fold in hypoxic cells (Fig. 15B). Three copies of the 26-base pair mPFK1 IVS-1 oligonucleotide were also cloned 5' to the psvcat transcription unit, and pPFKL3svcat mediated a 47-fold induction in hypoxic cells (Fig. 15B).

We also performed experiments with hALDA gene sequences to analyze native promoter function and to correlate sequence requirements for induction in

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the transfection assay with endogenous RNA expression data. The plasmid pNMHcat (Concordet et al. (1991) supra), in which 3.5 kb from the 5'-end of hALDA (Maire et al. (1987) supra) was fused to CAT coding sequences (Fig. 15A), mediated a 5.5-fold induction in hypoxic cells (Fig. 15B). The plasmid pHcat contained 0.76 kb of hALDA sequences that are colinear with the 3'-end of pNMHcat, starting within IVS-4 and extending 5' to exon H (Fig. 15A). Deletion of exons N1, N2, and M and their flanking sequences resulted in 20-fold increased levels of CAT expression but had no significant effect on relative expression in 1% O<sub>2</sub>, as pHcat was induced 5.4-fold in hypoxic Hep3B cells (Fig. 15B). These results are consistent with the observation of (i) specific induction of hALDA transcripts initiated from exon H and (ii) the presence of a HIF-1 binding site at the 5' end of IVS-4 contained within both pNMHcat and pHcat. Thus, sequences containing HIF-1 sites from the mPFKL, hPGK1, and hALDA genes mediated hypoxia-inducible transcription in conjunction with either a native or heterologous promoter.

**Example 10.      Construction of a Dominant-Negative Variant of HIF-1 $\alpha$ .**

A HIF-1 $\alpha$  variant was constructed to investigate functional inactivation of HIF-1.

The starting construct was the HIF-1 $\alpha$  cDNA 3.2-3 cloned into the plasmid pBluescript SK-. This plasmid was digested with the restriction endonucleases NcoI and BglII to delete sequences encoding amino acids 2-28. A double-stranded oligonucleotide was inserted that contained NcoI and BglII ends to allow recirculation of the plasmid in the presence of T4 DNA ligase. The resulting construct encodes amino acids 1-3, followed by three amino acids not present in the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, alanine, and glycine), followed by amino acids 28-826 of HIF-1 $\alpha$ . This construction (pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB) allows the *in vitro* transcription (using T7 RNA polymerase) and translation of the variant form of HIF-1 $\alpha$  (HIF-1 $\alpha$  $\Delta$ NB) (SEQ ID NO:35).

To create a dominant negative form of HIF-1 $\alpha$  for expression in mammalian tissue culture cells, a Kpn I-Not I fragment encoding the variant cDNA was excised from the pBluescript vector and cloned into the mammalian expression vector pCEP4. The plasmid was digested with AflII and BamHI, treated with Klenow form of DNA polymerase to generate blunt ends, and recircularized with

T4 DNA ligase. The resulting plasmid (pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB) (SEQ ID NO:3) encodes amino acids 1-3, followed by three amino acids not present at the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, alanine, and glycine), followed by amino acids 28-391 of HIF-1 $\alpha$ , followed by three amino acids not present at the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, glutamine, and threonine). Amino acids 392-826 were deleted to increase the stability of the variant protein (HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB) expressed in cells (Fig. 16).

Results. Hep3B cells were transiently transfected with 25 ug of the reporter gene psvcatEPO2 which contains two copies of the 33-bp enhancer sequence from the human erythropoietin gene as described above. This plasmid expressed a 9-fold higher level of CAT protein when cells were cultured at 1% O<sub>2</sub> relative to 20% O<sub>2</sub>. When the cells were transfected with psvcatEPO2 and pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB, there was dose-dependent inhibition of CAT expression at 1% O<sub>2</sub>. Table 3 shows the relative induction (expression at 1% O<sub>2</sub> divided by expression at 20% O<sub>2</sub>) as a function of the amount of pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB (ug) transfected into the cells. Results are the mean of three experiments.

Expression of variant HIF-1 $\alpha$  interfered with the activation of reporter gene expression by endogenous HIF-1 produced by hypoxic cells. The residual activation seen with 40 ug variant transfection may represent cells which took up psvcatEPO2 but not pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB. The results show that the dominant-negative variant can interfere with HIF-1 function *in vivo*.

The variant protein was used in a electrophoretic mobility shift assay of binding to a double-stranded oligonucleotide probe containing the HIF-1 binding site from the EPO enhancer. pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB was used as a template for *in vitro* transcription and translation. As increasing amounts of pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB were added to reactions containing a constant amount of templates for wild-type HIF-1 $\alpha$  and HIF-1 $\beta$ , there was a dose-dependent inhibition of DNA-binding such that when pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB was present in a 16-fold excess over the wild-type template pBluescript/HIF-1 $\alpha$ 3.2T7, HIF-1 DNA-binding was eliminated.

These *in vitro* and *in vivo* experiments demonstrate that deletion of the basic domain of HIF-1 $\alpha$  results in a protein that can block HIF-1 activity by inhibiting DNA binding.

TABLE 1. BIOCHEMICAL PURIFICATION OF HIF-1

Purification Step	Volume (ml)	Protein (mg)	HIF-1 Activity	Specific Activity	Yield (%)	Purification
HeLa nuclear Extract	435	3,040	608,000	0.2 U/ug	100	1
DEAE-Sepharose	240	550	440,000	0.8 U/mg	72	4
W18 DNA affinity 1	25	2.5	400,000	160	66	800
M18 DNA column	40	1.4	226,000	190	44	950
W18 DNA affinity 2	9	0.06	135,000	2,250	22	11,250

TABLE 2. OLIGONUCLEOTIDE SEQUENCES FROM EPO AND GLYCOLYTIC ENZYME GENES.

SEQUENCE	LOCATION	COORDINATES
gccc TACGTGCT gtctcacacagcctgtctga	hEPO 3'-FS	+3065/+3097
ccgggtagctggcg TACGTGCT gcag	mPFKL IVS-1	+336/+361
ggggctgctgca GACGTGCG tgtg	hEPO 5'-FS	-155/-178
gtga GACGTGCG gcttccgtttg	hPGK1 5'-FS	-172/-194
ctgcc GACGTGCG ctccggag	hPGK1 5'-UT	+31/+11
gtgggagcccagcg GACGTGCG ggaa	mLDHA 5'-FS	-75/-50
ggc CADGTGCG ccgcctgcgcctgcg	hENO1 5'-FS	-585/-610
ctc CACGTGCG gggaccagggaccgt	hALDA IVS-4	+125/+150

TABLE 3. RELATIVE INDUCTION OF REPORTER GENE IN THE PRESENCE OF HIF-1 $\alpha$  VARIANT.

ug Variant	Relative Hypoxic Induction
0	9.09
5	6.06
10	4.10
20	2.81
40	2.31

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: The Johns Hopkins University School of Medicine

(ii) TITLE OF INVENTION: HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE

5 (iii) NUMBER OF SEQUENCES: 35

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Fish & Richardson P.C.  
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 (C) CITY: La Jolla  
 (D) STATE: CA  
 (E) COUNTRY: USA  
 (F) ZIP: 92037

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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20 (A) APPLICATION NUMBER: PCT/US96/  
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(viii) ATTORNEY/AGENT INFORMATION:

25 (A) NAME: Haile, Lisa A.  
 (B) REGISTRATION NUMBER: 38,347  
 (C) REFERENCE/DOCKET NUMBER: 07265/053W01

(ix) TELECOMMUNICATION INFORMATION:

30 (A) TELEPHONE: 619/678-5070  
 (B) TELEFAX: 619/678-5099

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 3736 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

40	GTGAAGACAT CGCGGGGACC GATTCACC ATG GAG GGC GCC GGC GGC GCG AAC	52
	Met Glu Gly Ala Gly Gly Ala Asn	
	1 5	
	GAC AAG AAA AAG ATA AGT TCT GAA CGT CGA AAA GAA AAG TCT CGA GAT	100
	Asp Lys Lys Lys Ile Ser Ser Glu Arg Arg Lys Glu Lys Ser Arg Asp	
	10 15 20	
45	GCA GCC AGA TCT CGG CGA AGT AAA GAA TCT GAA GTT TTT TAT GAG CTT	148
	Ala Ala Arg Ser Arg Arg Ser Lys Glu Ser Glu Val Phe Tyr Glu Leu	
	25 30 35 40	

- 50 -

	GCT CAT CAG TTG CCA CTT CCA CAT AAT GTG AGT TCG CAT CTT GAT AAG Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His Leu Asp Lys 45 50 55	196
5	GCC TCT GTG ATG AGG CTT ACC ATC AGC TAT TTG CGT GTG AGG AAA CTT Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val Arg Lys Leu 60 65 70	244
	CTG GAT GCT GGT GAT TTG GAT ATT GAA GAT GAC ATG AAA GCA CAG ATG Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys Ala Gln Met 75 80 85	292
10	AAT TGC TTT TAT TTG AAA GCC TTG GAT GGT TTT GTT ATG GTT CTC ACA Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met Val Leu Thr 90 95 100	340
15	GAT GAT GGT GAC ATG ATT TAC ATT TCT GAT AAT GTG AAC AAA TAC ATG Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn Lys Tyr Met 105 110 115 120	388
	GGA TTA ACT CAG TTT GAA CTA ACT GGA CAC AGT GTG TTT GAT TTT ACT Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe Asp Phe Thr 125 130 135	436
20	CAT CCA TGT GAC CAT GAG GAA ATG AGA GAA ATG CTT ACA CAC AGA AAT His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr His Arg Asn 140 145 150	484
	GGC CTT GTG AAA AAG GGT AAA GAA CAA AAC ACA CAG CGA AGC TTT TTT Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg Ser Phe Phe 155 160 165	532
25	CTC AGA ATG AAG TGT ACC CTA ACT AGC CGA GGA AGA ACT ATG AAC ATA Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr Met Asn Ile 170 175 180	580
30	AAG TCT GCA ACA TGG AAG GTA TTG CAC TGC ACA GGC CAC ATT CAC GTA Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His Ile His Val 185 190 195 200	628
	TAT GAT ACC AAC AGT AAC CAA CCT CAG TGT GGG TAT AAG AAA CCA CCT Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys Lys Pro Pro 205 210 215	676
35	ATG ACC TGC TTG GTG CTG ATT TGT GAA CCC ATT CCT CAC CCA TCA AAT Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His Pro Ser Asn 220 225 230	724
	ATT GAA ATT CCT TTA GAT AGC AAG ACT TTC CTC AGT CGA CAC AGC CTG Ile Glu Ile Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg His Ser Leu 235 240 245	772
40	GAT ATG AAA TTT TCT TAT TGT GAT GAA AGA ATT ACC GAA TTG ATG GGA Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu Leu Met Gly 250 255 260	820
45	TAT GAG CCA GAA GAA CTT TTA GGC CGC TCA ATT TAT GAA TAT TAT CAT Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu Tyr Tyr His 265 270 275 280	868



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	GCT TTG GAC TCT GAT CAT CTG ACC AAA ACT CAT CAT GAT ATG TTT ACT	916
	Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp Met Phe Thr	
	285 290 295	
5	AAA GGA CAA GTC ACC ACA GGA CAG TAC AGG ATG CTT GCC AAA AGA GGT	964
	Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala Lys Arg Gly	
	300 305 310	
	GGA TAT GTC TGG GTT GAA ACT CAA GCA ACT GTC ATA TAT AAC ACC AAG	1012
	Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr Asn Thr Lys	
	315 320 325	
10	AAT TCT CAA CCA CAG TGC ATT GTA TGT GTG AAT TAC GTT GTG AGT GGT	1060
	Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val Val Ser Gly	
	330 335 340	
15	ATT ATT CAG CAC GAC TTG ATT TTC TCC CTT CAA CAA ACA GAA TGT GTC	1108
	Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr Glu Cys Val	
	345 350 355 360	
	CTT AAA CCG GTT GAA TCT TCA GAT ATG AAA ATG ACT CAG CTA TTC ACC	1156
	Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln Leu Phe Thr	
	365 370 375	
20	AAA GTT GAA TCA GAA GAT ACA AGT AGC CTC TTT GAC AAA CTT AAG AAG	1204
	Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys Leu Lys Lys	
	380 385 390	
	GAA CCT GAT GCT TTA ACT TTG CTG GCC CCA GCC GCT GGA GAC ACA ATC	1252
	Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly Asp Thr Ile	
	395 400 405	
25	ATA TCT TTA GAT TTT GGC AGC AAC GAC ACA GAA ACT GAT GAC CAG CAA	1300
	Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp Asp Gln Gln	
	410 415 420	
30	CTT GAG GAA GTA CCA TTA TAT AAT GAT GTA ATG CTC CCC TCA CCC AAC	1348
	Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro Ser Pro Asn	
	425 430 435 440	
	GAA AAA TTA CAG AAT ATA AAT TTG GCA ATG TCT CCA TTA CCC ACC GCT	1396
	Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu Pro Thr Ala	
	445 450 455	
35	GAA ACG CCA AAG CCA CTT CGA AGT AGT GCT GAC CCT GCA CTC AAT CAA	1444
	Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala Leu Asn Gln	
	460 465 470	
	GAA GTT GCA TTA AAA TTA GAA CCA AAT CCA GAG TCA CTG GAA CTT TCT	1492
	Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu Glu Leu Ser	
	475 480 485	
40	TTT ACC ATG CCC CAG ATT CAG GAT CAG ACA CCT AGT CCT TCC GAT GGA	1540
	Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro Ser Asp Gly	
	490 495 500	
45	AGC ACT AGA CAA AGT TCA CCT GAG CCT AAT AGT CCC AGT GAA TAT TGT	1588
	Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser Glu Tyr Cys	
	505 510 515 520	

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	TTT TAT GTG GAT AGT GAT ATG GTC AAT GAA TTC AAG TTG GAA TTG GTA Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val 525 530 535	1636
5	GAA AAA CTT TTT GCT GAA GAC ACA GAA GCA AAG AAC CCA TTT TCT ACT Glu Lys Leu Thr Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr 540 545 550	1684
	CAG GAC ACA GAT TTA GAC TTG GAG ATG TTA GCT CCC TAT ATC CCA ATG Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met 555 560 565	1732
10	GAT GAT GAC TTC CAG TTA CGT TCC TTC GAT CAG TTG TCA CCA TTA GAA Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu 570 575 580	1780
15	AGC AGT TCC GCA AGC CCT GAA AGC GCA AGT CCT CAA AGC ACA GTT ACA Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr 585 590 595 600	1828
	GTA TTC CAG CAG ACT CAA ATA CAA GAA CCT ACT GCT AAT GCC ACC ACT Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr 605 610 615	1876
20	ACC ACT GCC ACC ACT GAT GAA TTA AAA ACA GTG ACA AAA GAC CGT ATG Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met 620 625 630	1924
	GAA GAC ATT AAA ATA TTG ATT GCA TCT CCA TCT CCT ACC CAC ATA CAT Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr His Ile His 635 640 645	1972
25	AAA GAA ACT ACT AGT GCC ACA TCA TCA CCA TAT AGA GAT ACT CAA AGT Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp Thr Gln Ser 650 655 660	2020
30	CGG ACA GCC TCA CCA AAC AGA GCA GGA AAA GGA GTC ATA GAA CAG ACA Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile Glu Gln Thr 665 670 675 680	2068
	GAA AAA TCT CAT CCA AGA AGC CCT AAC GTG TTA TCT GTC GCT TTG AGT Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val Ala Leu Ser 685 690 695	2116
35	CAA AGA ACT ACA GTT CCT GAG GAA GAA CTA AAT CCA AAG ATA CTA GCT Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys Ile Leu Ala 700 705 710	2164
	TTG CAG AAT GCT CAG AGA AAG CGA AAA ATG GAA CAT GAT GGT TCA CTT Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp Gly Ser Leu 715 720 725	2212
40	TTT CAA GCA GTA GGA ATT GGA ACA TTA TTA CAG CAG CCA GAC GAT CAT Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Pro Asp Asp His 730 735 740	2260
45	GCA GCT ACT ACA TCA CTT TCT TGG AAA CGT GTA AAA GGA TGC AAA TCT Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly Cys Lys Ser 745 750 755 760	2308

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	AGT GAA CAG AAT GGA ATG GAG CAA AAG ACA ATT ATT TTA ATA CCC TCT	2356
	Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu Ile Pro Ser	
	765 770 775	
5	GAT TTA GCA TGT AGA CTG CTG GGG CAA TCA ATG GAT GAA AGT GGA TTA	2404
	Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu Ser Gly Leu	
	780 785 790	
	CCA CAG CTG ACC AGT TAT GAT TGT GAA GTT AAT GCT CCT ATA CAA GGC	2452
	Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln Gly	
	795 800 805	
10	AGC AGA AAC CTA CTG CAG GGT GAA GAA TTA CTC AGA GCT TTG GAT CAA	2500
	Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala Leu Asp Gln	
	810 815 820	
15	GTT AAC T GAGCTTTTTT TTAATTTTCAT TCCTTTTTTTT GGACACTGGT GGCTCACTAC	2557
	Val Asn	
	825	
	CTAAAGCAGT CTATTTATAT TTTCTACATC TAATTTTAGA AGCCTGGCTA CAATACTGCA	2617
	CAAACCTGGT TAGTTCAATT TTTGATCCCC TTTCTACTTA ATTTACATTA ATGCTCTTTT	2677
	TTAGTATGTT CTTTAATGCT GGATCACAGA CAGCTCATTT TCTCAGTTTT TTGGTATTTA	2737
	AACCATTGCA TTGCAGTAGC ATCATTAAAT AAAAAATGCA CCTTTTTATT TATTTATTTT	2797
20	TGGCTAGGGA GTTTATCCCT TTTTCGAATT ATTTTAAAGA AGATGCCAAT ATAATTTTTG	2857
	TAAGAAGGCA GTAACCTTTC ATCATGATCA TAGGCAGTTG AAAAAATTTT ACACCTTTTT	2917
	TTTCACAAAT TTTACATAAA TAATAATGCT TTGCCAGCAG TACGTGGTAG CCACAATTGC	2977
	ACAATATATT TTCTTAAAAA ATACCAGCAG TTAATCATGG AATATATTCT GCGTTTATAA	3037
	AACTAGTTTT TAAGAAGAAA TTTTTTTTGG CCTATGAAAT TGTTAAACAA CTGGAACATG	3097
25	ACATTGTTAA TCATATAATA ATGATTCTTA AATGCTGTAT GGTTTATTAT TTAAATGGGT	3157
	AAAGCCATTT ACATAATATA GAAAGATATG CATATATCTA GAAGGTATGT GGCATTATT	3217
	TGGATAAAAT TCTCAATTCA GAGAAATCAA ATCTGATGTT TCTATAGTCA CTTTGCCAGC	3277
	TCAAAAGAAA ACAATACCCT ATGTAGTTGT GGAAGTTTAT GCTAATATTG TGTAAGTATG	3337
	ATTAAACCTA AATGTTCTGC CTACCCTGTT GGTATAAAGA TATTTTGAGC AGACTGTAAA	3397
30	CAAGAAAAAA AAAAAATCAT GCATTCTTAG CAAAATTGCC TAGTATGTTA ATTTGCTCAA	3457
	AATACAATGT TTGATTTTAT GCACTTTGTC GCTATTAAAC TCCTTTTTTT CATGTAGATT	3517
	TCAATAATTG AGTAATTTTA GAAGCATTAT TTTAGGAATA TATAGTTGTC AAAACAGTA	3577
	AATATCTTGT TTTTCTATG TACATTGTAC AAATTTTTC TTTCTTTTGC TCTTTGTGGT	3637
	TGGATCTAAC ACTAAGTGA TTGTTTTGTT ACATCAAATA AACATCTTCT GTGGAAAAAA	3697
35	AAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	3736

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 826 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

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## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu  
 1 5 10 15

Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys  
 20 25 30

Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His  
 35 40 45

Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile  
 50 55 60

Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile  
 65 70 75 80

Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu  
 85 90 95

Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile  
 100 105 110

Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr  
 115 120 125

Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met  
 130 135 140

Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu  
 145 150 155 160

Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr  
 165 170 175

Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu  
 180 185 190

His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro  
 195 200 205

Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys  
 210 215 220

Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys  
 225 230 235 240

Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp  
 245 250 255

Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly  
 260 265 270

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	Arg	Ser	Ile	Tyr	Glu	Tyr	Tyr	His	Ala	Leu	Asp	Ser	Asp	His	Leu	Thr	
			275					280					285				
	Lys	Thr	His	His	Asp	Met	Phe	Thr	Lys	Gly	Gln	Val	Thr	Thr	Gly	Gln	
			290				295					300					
5	Tyr	Arg	Met	Leu	Ala	Lys	Arg	Gly	Gly	Tyr	Val	Trp	Val	Glu	Thr	Gln	
	305					310					315					320	
	Ala	Thr	Val	Ile	Tyr	Asn	Thr	Lys	Asn	Ser	Gln	Pro	Gln	Cys	Ile	Val	
					325					330					335		
10	Cys	Val	Asn	Tyr	Val	Val	Ser	Gly	Ile	Ile	Gln	His	Asp	Leu	Ile	Phe	
			340						345					350			
	Ser	Leu	Gln	Gln	Thr	Glu	Cys	Val	Leu	Lys	Pro	Val	Glu	Ser	Ser	Asp	
			355					360					365				
	Met	Lys	Met	Thr	Gln	Leu	Phe	Thr	Lys	Val	Glu	Ser	Glu	Asp	Thr	Ser	
		370					375					380					
15	Ser	Leu	Phe	Asp	Lys	Leu	Lys	Lys	Glu	Pro	Asp	Ala	Leu	Thr	Leu	Leu	
	385					390					395					400	
	Ala	Pro	Ala	Ala	Gly	Asp	Thr	Ile	Ile	Ser	Leu	Asp	Phe	Gly	Ser	Asn	
					405					410					415		
20	Asp	Thr	Glu	Thr	Asp	Asp	Gln	Gln	Leu	Glu	Glu	Val	Pro	Leu	Tyr	Asn	
			420						425					430			
	Asp	Val	Met	Leu	Pro	Ser	Pro	Asn	Glu	Lys	Leu	Gln	Asn	Ile	Asn	Leu	
			435					440					445				
	Ala	Met	Ser	Pro	Leu	Pro	Thr	Ala	Glu	Thr	Pro	Lys	Pro	Leu	Arg	Ser	
		450					455					460					
25	Ser	Ala	Asp	Pro	Ala	Leu	Asn	Gln	Glu	Val	Ala	Leu	Lys	Leu	Glu	Pro	
	465					470					475					480	
	Asn	Pro	Glu	Ser	Leu	Glu	Leu	Ser	Phe	Thr	Met	Pro	Gln	Ile	Gln	Asp	
					485					490					495		
30	Gln	Thr	Pro	Ser	Pro	Ser	Asp	Gly	Ser	Thr	Arg	Gln	Ser	Ser	Pro	Glu	
			500						505						510		
	Pro	Asn	Ser	Pro	Ser	Glu	Tyr	Cys	Phe	Tyr	Val	Asp	Ser	Asp	Met	Val	
			515					520					525				
	Asn	Glu	Phe	Lys	Leu	Glu	Leu	Val	Glu	Lys	Leu	Phe	Ala	Glu	Asp	Thr	
		530					535					540					
35	Glu	Ala	Lys	Asn	Pro	Phe	Ser	Thr	Gln	Asp	Thr	Asp	Leu	Asp	Leu	Glu	
	545					550					555					560	
	Met	Leu	Ala	Pro	Tyr	Ile	Pro	Met	Asp	Asp	Asp	Phe	Gln	Leu	Arg	Ser	
					565					570					575		
40	Phe	Asp	Gln	Leu	Ser	Pro	Leu	Glu	Ser	Ser	Ser	Ala	Ser	Pro	Glu	Ser	
				580					585					590			

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Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln  
 595 600 605  
 Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu  
 610 615 620  
 5 Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala  
 625 630 635 640  
 Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser  
 645 650 655  
 10 Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala  
 660 665 670  
 Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro  
 675 680 685  
 Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu  
 690 695 700  
 15 Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg  
 705 710 715 720  
 Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr  
 725 730 735  
 20 Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp  
 740 745 750  
 Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln  
 755 760 765  
 Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly  
 770 775 780  
 25 Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys  
 785 790 795 800  
 Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu  
 805 810 815  
 30 Glu Leu Leu Arg Ala Leu Asp Gln Val Asn  
 820 825  
 (2) INFORMATION FOR SEQ ID NO:3:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 373 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
 40 Met Glu Gly Ile Ala Gly Ser Arg Arg Ser Lys Glu Ser Glu Val Phe  
 1 5 10 15

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	Tyr	Glu	Leu	Ala	His	Gln	Leu	Pro	Leu	Pro	His	Asn	Val	Ser	Ser	His	
				20					25					30			
	Leu	Asp	Lys	Ala	Ser	Val	Met	Arg	Leu	Thr	Ile	Ser	Tyr	Leu	Arg	Val	
			35					40					45				
5	Arg	Lys	Leu	Leu	Asp	Ala	Gly	Asp	Leu	Asp	Ile	Glu	Asp	Asp	Met	Lys	
		50					55					60					
	Ala	Gln	Met	Asn	Cys	Phe	Tyr	Leu	Lys	Ala	Leu	Asp	Gly	Phe	Val	Met	
	65				70					75						80	
10	Val	Leu	Thr	Asp	Asp	Gly	Asp	Met	Ile	Tyr	Ile	Ser	Asp	Asn	Val	Asn	
				85						90					95		
	Lys	Tyr	Met	Gly	Leu	Thr	Gln	Phe	Glu	Leu	Thr	Gly	His	Ser	Val	Phe	
			100						105					110			
	Asp	Phe	Thr	His	Pro	Cys	Asp	His	Glu	Glu	Met	Arg	Glu	Met	Leu	Thr	
			115					120					125				
15	His	Arg	Asn	Gly	Leu	Val	Lys	Lys	Gly	Lys	Glu	Gln	Asn	Thr	Gln	Arg	
		130					135					140					
	Ser	Phe	Phe	Leu	Arg	Met	Lys	Cys	Thr	Leu	Thr	Ser	Arg	Gly	Arg	Thr	
	145				150						155					160	
20	Met	Asn	Ile	Lys	Ser	Ala	Thr	Trp	Lys	Val	Leu	His	Cys	Thr	Gly	His	
				165						170					175		
	Ile	His	Val	Tyr	Asp	Thr	Asn	Ser	Asn	Gln	Pro	Gln	Cys	Gly	Tyr	Lys	
			180						185					190			
	Lys	Pro	Pro	Met	Thr	Cys	Leu	Val	Leu	Ile	Cys	Glu	Pro	Ile	Pro	His	
			195					200					205				
25	Pro	Ser	Asn	Ile	Glu	Ile	Pro	Leu	Asp	Ser	Lys	Thr	Phe	Leu	Ser	Arg	
		210					215					220					
	His	Ser	Leu	Asp	Met	Lys	Phe	Ser	Tyr	Cys	Asp	Glu	Arg	Ile	Thr	Glu	
	225				230					235						240	
30	Leu	Met	Gly	Tyr	Glu	Pro	Glu	Glu	Leu	Leu	Gly	Arg	Ser	Ile	Tyr	Glu	
				245					250						255		
	Tyr	Tyr	His	Ala	Leu	Asp	Ser	Asp	His	Leu	Thr	Lys	Thr	His	His	Asp	
			260						265					270			
	Met	Phe	Thr	Lys	Gly	Gln	Val	Thr	Thr	Gly	Gln	Tyr	Arg	Met	Leu	Ala	
			275					280					285				
35	Lys	Arg	Gly	Gly	Tyr	Val	Trp	Val	Glu	Thr	Gln	Ala	Thr	Val	Ile	Tyr	
		290					295					300					
	Asn	Thr	Lys	Asn	Ser	Gln	Pro	Gln	Cys	Ile	Val	Cys	Val	Asn	Tyr	Val	
	305				310						315					320	
40	Val	Ser	Gly	Ile	Ile	Gln	His	Asp	Leu	Ile	Phe	Ser	Leu	Gln	Gln	Thr	
				325						330					335		

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Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln  
 340 345 350

Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys  
 355 360 365

5 Leu Lys Ile Gln Thr  
 370

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 805 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 Met Glu Gly Ile Ala Gly Ser Arg Arg Ser Lys Glu Ser Glu Val Phe  
 1 5 10 15

Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His  
 20 25 30

20 Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val  
 35 40 45

Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys  
 50 55 60

Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met  
 65 70 75 80

25 Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn  
 85 90 95

Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe  
 100 105 110

30 Asp Phe Thr His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr  
 115 120 125

His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg  
 130 135 140

Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr  
 145 150 155 160

35 Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His  
 165 170 175

Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys  
 180 185 190

40 Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His  
 195 200 205



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	Pro Ser Asn Ile Glu Ile	Pro Leu Asp Ser Lys	Thr Phe Leu Ser Arg
	210	215	220
	His Ser Leu Asp Met Lys	Phe Ser Tyr Cys Asp	Glu Arg Ile Thr Glu
	225	230	235 240
5	Leu Met Gly Tyr Glu Pro	Glu Glu Leu Leu Gly	Arg Ser Ile Tyr Glu
	245	250	255
	Tyr Tyr His Ala Leu Asp	Ser Asp His Leu Thr	Lys Thr His His Asp
	260	265	270
10	Met Phe Thr Lys Gly Gln	Val Thr Thr Gly Gln	Tyr Arg Met Leu Ala
	275	280	285
	Lys Arg Gly Gly Tyr Val	Trp Val Glu Thr Gln	Ala Thr Val Ile Tyr
	290	295	300
	Asn Thr Lys Asn Ser Gln	Pro Gln Cys Ile Val	Cys Val Asn Tyr Val
	305	310	315 320
15	Val Ser Gly Ile Ile Gln	His Asp Leu Ile Phe	Ser Leu Gln Gln Thr
	325	330	335
	Glu Cys Val Leu Lys Pro	Val Glu Ser Ser Asp	Met Lys Met Thr Gln
	340	345	350
20	Leu Phe Thr Lys Val Glu	Ser Glu Asp Thr Ser	Ser Leu Phe Asp Lys
	355	360	365
	Leu Lys Lys Glu Pro Asp	Ala Leu Thr Leu Leu	Ala Pro Ala Ala Gly
	370	375	380
	Asp Thr Ile Ile Ser Leu	Asp Phe Gly Ser Asn	Asp Thr Glu Thr Asp
	385	390	395 400
25	Asp Gln Gln Leu Glu Glu	Val Pro Leu Tyr Asn	Asp Val Met Leu Pro
	405	410	415
	Ser Pro Asn Glu Lys Leu	Gln Asn Ile Asn Leu	Ala Met Ser Pro Leu
	420	425	430
30	Pro Thr Ala Glu Thr Pro	Lys Pro Leu Arg Ser	Ser Ala Asp Pro Ala
	435	440	445
	Leu Asn Gln Glu Val Ala	Leu Lys Leu Glu Pro	Asn Pro Glu Ser Leu
	450	455	460
	Glu Leu Ser Phe Thr Met	Pro Gln Ile Gln Asp	Gln Thr Pro Ser Pro
	465	470	475 480
35	Ser Asp Gly Ser Thr Arg	Gln Ser Ser Pro Glu	Pro Asn Ser Pro Ser
	485	490	495
	Glu Tyr Cys Phe Tyr Val	Asp Ser Asp Met Val	Asn Glu Phe Lys Leu
	500	505	510
40	Glu Leu Val Glu Lys Leu	Phe Ala Glu Asp Thr	Glu Ala Lys Asn Pro
	515	520	525

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	Phe	Ser	Thr	Gln	Asp	Thr	Asp	Leu	Asp	Leu	Glu	Met	Leu	Ala	Pro	Tyr
	530						535					540				
	Ile	Pro	Met	Asp	Asp	Asp	Phe	Gln	Leu	Arg	Ser	Phe	Asp	Gln	Leu	Ser
	545					550					555					560
5	Pro	Leu	Glu	Ser	Ser	Ser	Ala	Ser	Pro	Glu	Ser	Ala	Ser	Pro	Gln	Ser
					565					570					575	
	Thr	Val	Thr	Val	Phe	Gln	Gln	Thr	Gln	Ile	Gln	Glu	Pro	Thr	Ala	Asn
				580					585					590		
10	Ala	Thr	Thr	Thr	Thr	Ala	Thr	Thr	Asp	Glu	Leu	Lys	Thr	Val	Thr	Lys
			595					600					605			
	Asp	Arg	Met	Glu	Asp	Ile	Lys	Ile	Leu	Ile	Ala	Ser	Pro	Ser	Pro	Thr
		610					615					620				
	His	Ile	His	Lys	Glu	Thr	Thr	Ser	Ala	Thr	Ser	Ser	Pro	Tyr	Arg	Asp
	625					630					635					640
15	Thr	Gln	Ser	Arg	Thr	Ala	Ser	Pro	Asn	Arg	Ala	Gly	Lys	Gly	Val	Ile
					645					650					655	
	Glu	Gln	Thr	Glu	Lys	Ser	His	Pro	Arg	Ser	Pro	Asn	Val	Leu	Ser	Val
				660					665					670		
20	Ala	Leu	Ser	Gln	Arg	Thr	Thr	Val	Pro	Glu	Glu	Glu	Leu	Asn	Pro	Lys
			675					680					685			
	Ile	Leu	Ala	Leu	Gln	Asn	Ala	Gln	Arg	Lys	Arg	Lys	Met	Glu	His	Asp
		690				695						700				
	Gly	Ser	Leu	Phe	Gln	Ala	Val	Gly	Ile	Gly	Thr	Leu	Leu	Gln	Gln	Pro
	705					710					715					720
25	Asp	Asp	His	Ala	Ala	Thr	Thr	Ser	Leu	Ser	Trp	Lys	Arg	Val	Lys	Gly
				725						730					735	
	Cys	Lys	Ser	Ser	Glu	Gln	Asn	Gly	Met	Glu	Gln	Lys	Thr	Ile	Ile	Leu
				740					745					750		
30	Ile	Pro	Ser	Asp	Leu	Ala	Cys	Arg	Leu	Leu	Gly	Gln	Ser	Met	Asp	Glu
			755					760					765			
	Ser	Gly	Leu	Pro	Gln	Leu	Thr	Ser	Tyr	Asp	Cys	Glu	Val	Asn	Ala	Pro
		770					775					780				
	Ile	Gln	Gly	Ser	Arg	Asn	Leu	Leu	Gln	Gly	Glu	Glu	Leu	Leu	Arg	Ala
	785					790					795					800
35	Leu	Asp	Gln	Val	Asn											
					805											

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- 40
- (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- 61 -

- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
- GATCGCCCTA CGTGCTGTCT CA 22
- (2) INFORMATION FOR SEQ ID NO:6:
- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
- GATCGCCCTA AAAGCTGTCT CA 22
- (2) INFORMATION FOR SEQ ID NO:7:
- 15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- 20 (ix) FEATURE:  
(D) OTHER INFORMATION: N at positions 15 and 27 is inosine.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
- ATCGGATCCA TCACNGARCT SATGGGNTAT A 31
- (2) INFORMATION FOR SEQ ID NO:8:
- 25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:  
(D) OTHER INFORMATION: N is inosine.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
- ATTAAGCMTG GTSAGGTGGT CNSWGTC 27
- (2) INFORMATION FOR SEQ ID NO:9:
- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 40

- 62 -

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTAAGCTTG CATGGTAGTA YTCATAGAT

29

(2) INFORMATION FOR SEQ ID NO:10:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(D) OTHER INFORMATION: N is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAAGCTTG TSTAYGTSTC NGAYTCGG

28

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(D) OTHER INFORMATION: N is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25

ATCGAATTCY TCNGACTGNG GCTGGTT

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACGGATCCG CCATGGCGGC GACTACTGA

29

35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

- 63 -

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCCAGGGCA CTACAGGTGG GTACC

25

(2) INFORMATION FOR SEQ ID NO:14:

5

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTCCCCGCA AGGACTTCAT GTGAG

25

(2) INFORMATION FOR SEQ ID NO:15:

15

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 15 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ile	Thr	Glu	Leu	Met	Gly	Tyr	Glu	Pro	Glu	Glu	Leu	Leu	Gly	Arg
1				5				10					15	

(2) INFORMATION FOR SEQ ID NO:16:

25

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Xaa	Ile	Ile	Leu	Ile	Pro	Ser	Asp	Leu	Ala	Xaa	Arg
1				5				10			

(2) INFORMATION FOR SEQ ID NO:17:

35

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 64 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser	Ile	Tyr	Glu	Tyr	Tyr	His	Ala	Leu	Asp	Ser	Asp	His	Leu	Thr	Lys
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:18:

5

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser	Phe	Phe	Leu	Arg
1			5	

(2) INFORMATION FOR SEQ ID NO:19:

15

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCRCCATGG

10

(2) INFORMATION FOR SEQ ID NO:20:

25

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCACCATGG

10

(2) INFORMATION FOR SEQ ID NO:21:

35

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

- 65 -

Val Val Tyr Val Ser Asp Ser Val Thr Pro Val Leu Asn Gln Pro Gln  
 1 5 10 15

Ser Glu

5 (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Thr Ser Gln Phe Gly Val Gly Ser Phe Gln Thr Pro Ser Ser Phe Ser  
 1 5 10 15

15

Ser Met Xaa Leu Pro Gly Ala Pro Thr Ala Ser Pro Gly Ala Ala Ala  
 20 25 30

Tyr

(2) INFORMATION FOR SEQ ID NO:23:

20

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 6 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CACGTG

6

(2) INFORMATION FOR SEQ ID NO:24:

30

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 7 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

BACGTGC

7

(2) INFORMATION FOR SEQ ID NO:25:

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- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:  
(D) OTHER INFORMATION: N is inosine.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
- 10 TNGNGCGTGM SA 12
- (2) INFORMATION FOR SEQ ID NO:26:
- 15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
- UUAUUUAAWW 9
- 20 (2) INFORMATION FOR SEQ ID NO:27:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
- ATAGGATCCT CAGGTCAGCT GGCACCCAG 29
- (2) INFORMATION FOR SEQ ID NO:28:
- 30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
- CCAAAGCTTC TATTCTGAAA AGGGGGG 27
- (2) INFORMATION FOR SEQ ID NO:29:



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- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

RWACGTG

7

- 10 (2) INFORMATION FOR SEQ ID NO:30:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TACGTGCT

8

- (2) INFORMATION FOR SEQ ID NO:31:
- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GACGTGCG

8

- (2) INFORMATION FOR SEQ ID NO:32:
- 30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

35 CACGTGCG

8

- (2) INFORMATION FOR SEQ ID NO:33:
- 40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

BACGTGCK

8

(2) INFORMATION FOR SEQ ID NO:34:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CACGTGCT

8

(2) INFORMATION FOR SEQ ID NO:35:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met	Glu	Gly	Ile	Ala	Gly	Ala	Asn	Asp	Lys	Lys	Lys	Ile	Ser	Ser	Glu
1				5				10					15		

Arg	Arg	Lys	Glu	Lys	Ser	Arg	Asp	Ala	Ala	Arg	Ser	Arg	Arg
			20				25					30	

25

## Claims

1. Purified human HIF-1.
2. The human HIF-1 $\alpha$  polypeptide encoded by
  - (a) the DNA sequence set out in Fig. 10 (SEQ ID NO:1) or its  
5 complementary strand; and
  - (b) DNA sequences which hybridize under stringent conditions to the  
DNA sequences defined in (a).
3. An isolated nucleotide sequence encoding the human HIF-1 $\alpha$   
polypeptide.
- 10 4. The isolated nucleotide sequence of claim 3 selected from the group  
consisting of:
  - (a) SEQ ID NO:1;
  - (b) nucleic acid sequences complementary to SEQ ID NO:1;
  - (c) fragments of (a) or (b) that are at least 15 bases in length and that will  
15 selectively hybridize to nucleotides which encode the HIF-1 $\alpha$  polypeptide of SEQ  
ID NO:1, under stringent conditions.
5. The nucleotide of claim 3, wherein the nucleotide is isolated from a  
mammalian cell.
6. The nucleotide of claim 5, wherein the mammalian cell is a human  
20 cell.
7. An expression vector including the nucleotide of claim 3.
8. The vector of claim 7, wherein the vector is a plasmid.
9. The vector of claim 7, wherein the vector is a virus.
10. A host cell stably transformed with the vector of claim 7.

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11. The host cell of claim 10, wherein the cell is prokaryotic.
12. The host cell of claim 10, wherein the cell is eukaryotic.
13. A purified antibody that binds to HIF-1 or to the HIF-1 $\alpha$  polypeptide or immunoreactive fragments thereof.
- 5 14. The antibody of claim 13, wherein the antibody is polyclonal.
15. The antibody of claim 13, wherein the antibody is monoclonal.
16. A purified and isolated nucleotide sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of HIF-1 $\alpha$  to allow possession of the biological activities of promoting the synthesis of erythropoietin (EPO), aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), pyruvate kinase M (PKM) and vascular endothelial growth factor (VEGF) in Hep3B cells.
- 10 17. A human HIF-1 $\alpha$  variant polypeptide which dimerizes with an HIF-1 $\beta$  isoform wherein at least one of the amino acids of SEQ ID NO:2 is replaced by another amino acid.
- 15 18. An isolated nucleotide sequence encoding the human variant HIF-1 $\alpha$  polypeptide having the sequence of SEQ ID NO:4.
19. A method of detecting HIF-1 $\alpha$  comprising contacting a specimen of a subject with a reagent that binds HIF-1 $\alpha$  and detecting binding of the reagent to HIF-1 $\alpha$ .
- 20 20. The method of claim 19 wherein the reagent is a nucleotide sequence complementary to SEQ ID NO:1 or a portion thereof.
21. The method of claim 18 wherein the reagent is an antibody specific for HIF-1 $\alpha$ .

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22. A method for enhancing expression of a structural genetic sequence whose regulatory region contains an HIF-1 binding site, comprising administering a therapeutically effective amount of a nucleotide sequence encoding HIF-1 $\alpha$ , whereby expression of the structural genetic sequence is enhanced.

5           23. The method of claim 22, wherein the structural genetic sequence encodes EPO.

24. The method of claim 22, wherein the structural genetic sequence encodes VEGF.

10           25. The method of claim 22, wherein the structural genetic sequence encodes a glycolytic enzyme.

26. A method of treating hypoxia-related tissue damage in a subject in need thereof, comprising administering a therapeutically effective amount of a nucleotide sequence encoding HIF-1 $\alpha$ , wherein tissue damage is substantially inhibited.

27. A method of treating hypoxia-related tissue damage in a subject in need thereof, comprising introducing a nucleotide sequence of claim 3 into cells of the subject, wherein a therapeutically effective amount of HIF-1 $\alpha$  is expressed in the subject, wherein tissue damage is substantially inhibited.

5           28. A method for inhibiting expression of a structural genetic sequence whose regulatory region contains an HIF-1 binding site, comprising administering a therapeutically effective amount of an inhibitory nucleotide sequence, whereby expression of the structural genetic sequence is inhibited.

10           29. The method of claim 28 wherein the inhibitory nucleotide sequence hybridizes to an HIF-1 $\alpha$  encoding nucleotide sequence.

30. The method of claim 29, wherein the HIF-1 $\alpha$  encoding nucleotide sequence is RNA.

31. The method of claim 29, wherein the HIF-1 $\alpha$  encoding nucleotide sequence is DNA.

15           32. The method of claim 28 wherein the inhibitory nucleotide sequence encodes an HIF-1 $\alpha$  variant polypeptide.

33. A pharmaceutical composition comprising a pharmaceutically acceptable carrier admixed with a therapeutically effective amount of HIF-1.

20           34. A pharmaceutical composition comprising a nucleotide sequence encoding HIF-1 $\alpha$  in a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising an HIF-1 $\alpha$  inhibitory nucleotide sequence in a pharmaceutically acceptable carrier.

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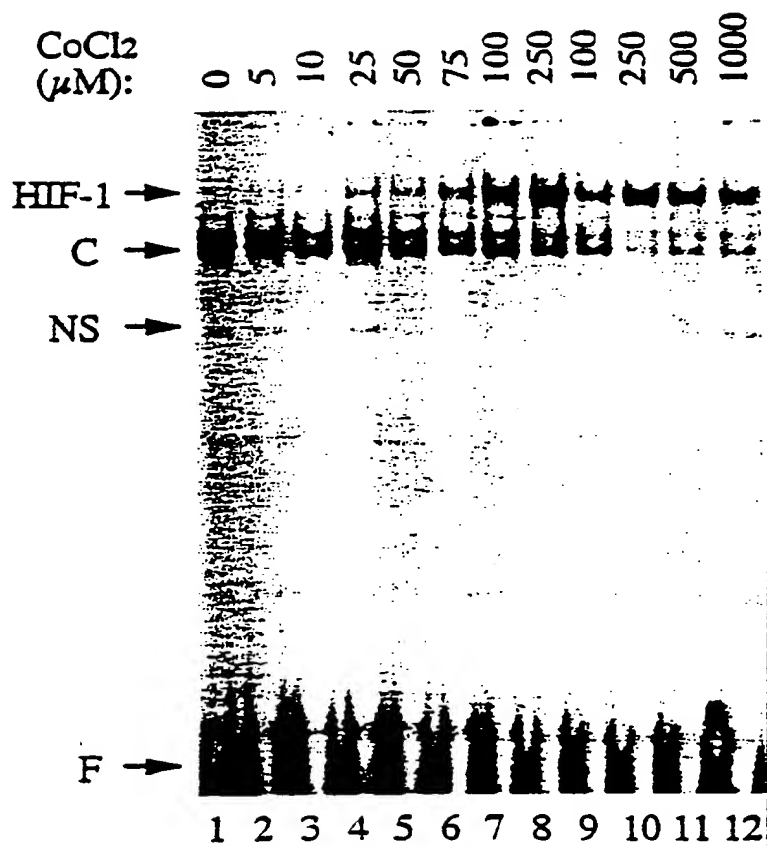


Figure 1

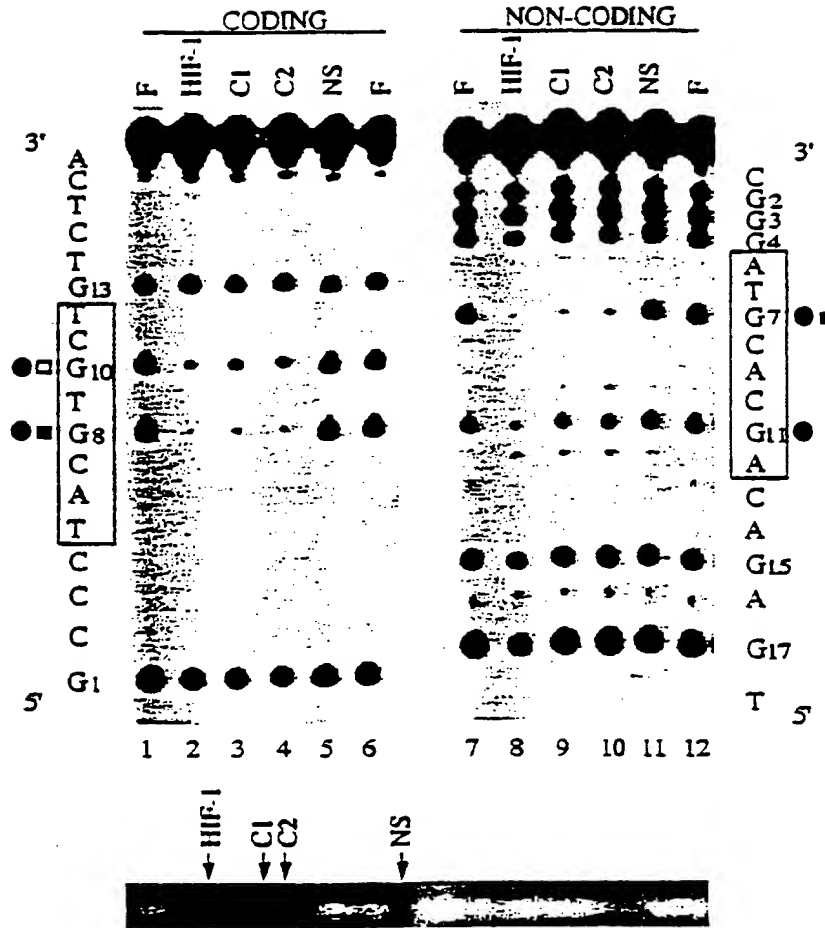


Figure 2



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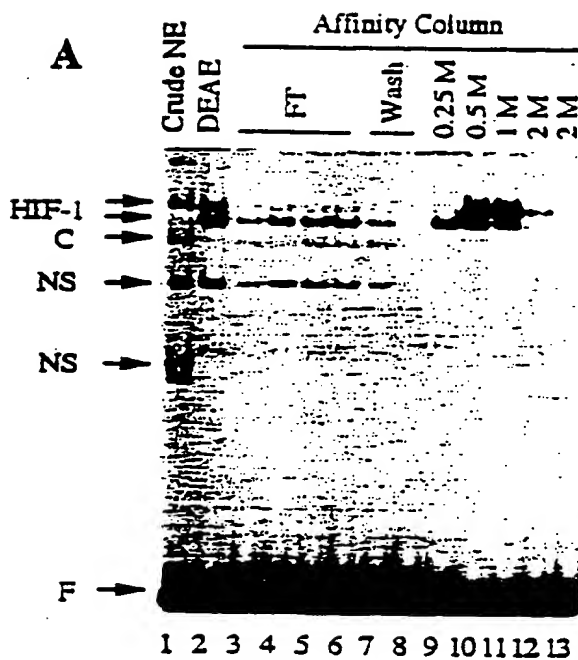


Figure 3A

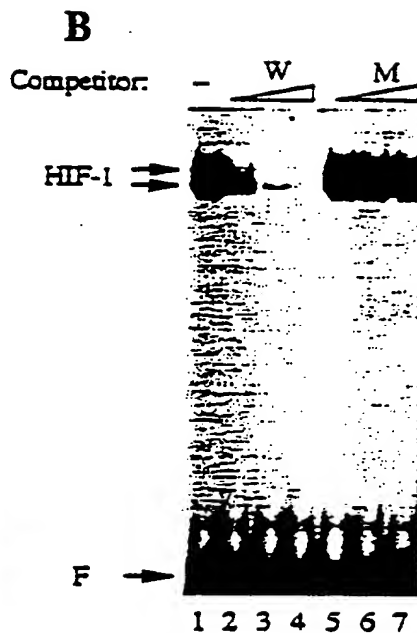


Figure 3B

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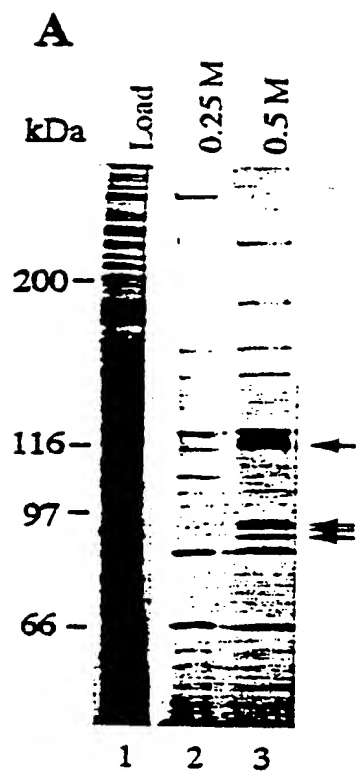


Figure 4A

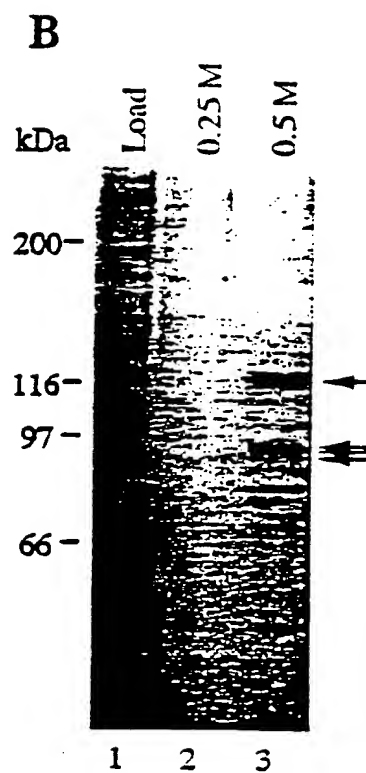


Figure 4B

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Figure 5 A

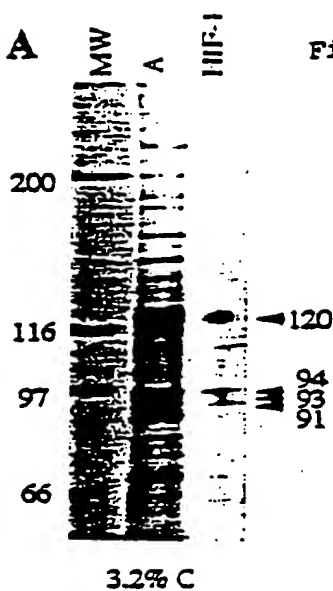


Figure 5 B

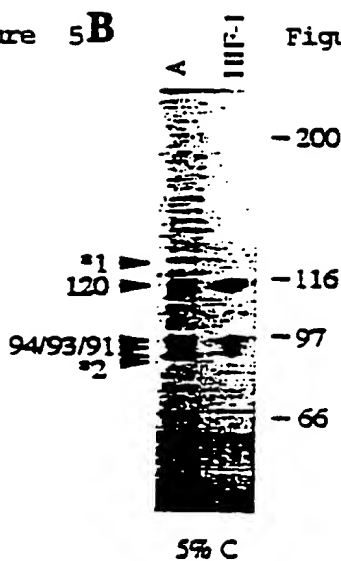
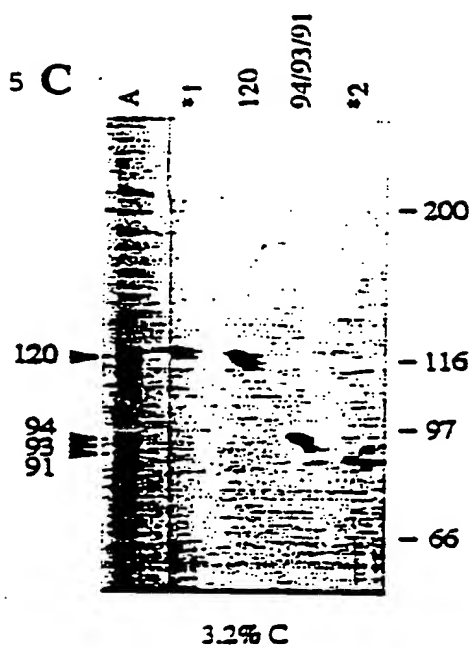


Figure 5 C



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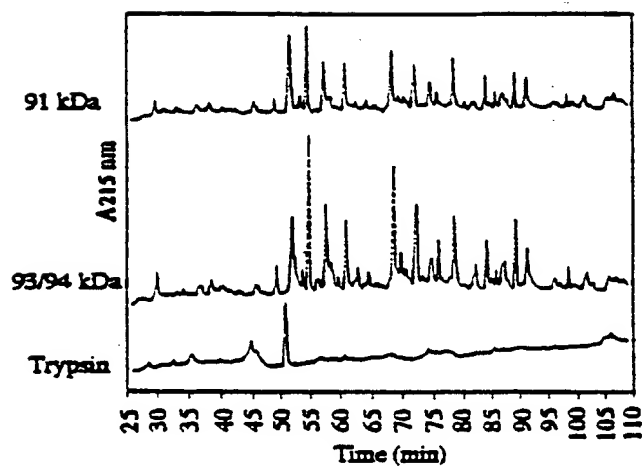


Figure 6

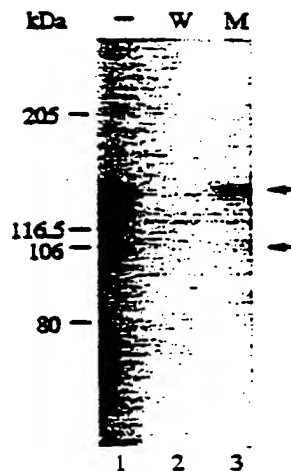


Figure 7

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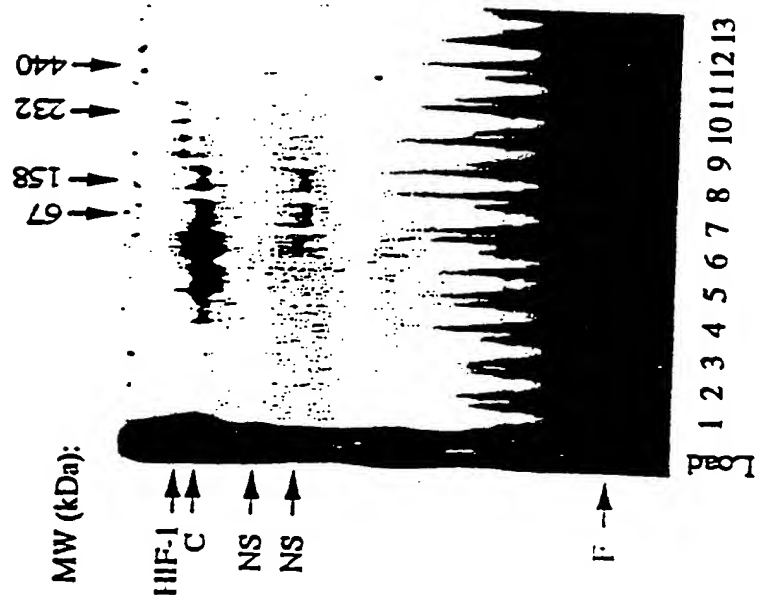


Figure 8

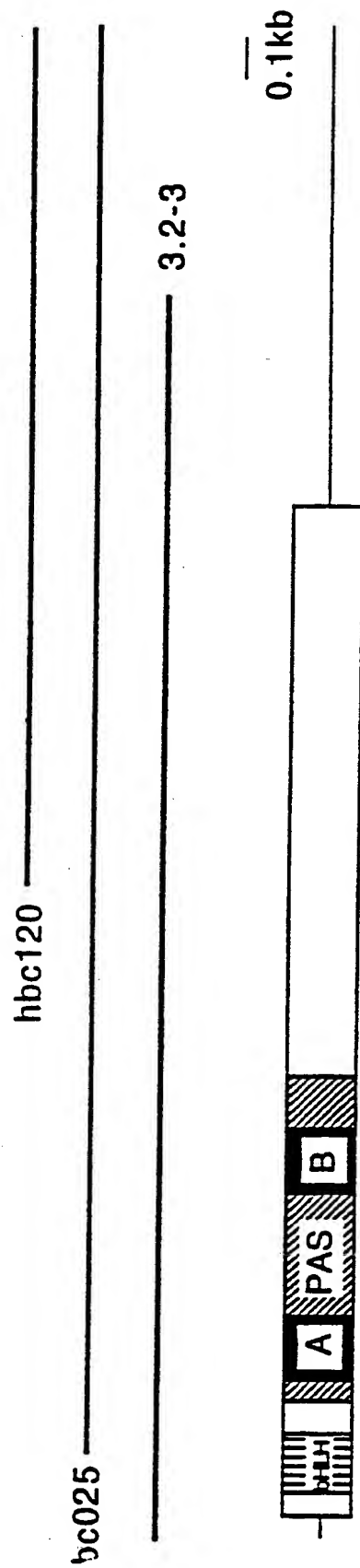


Figure 9

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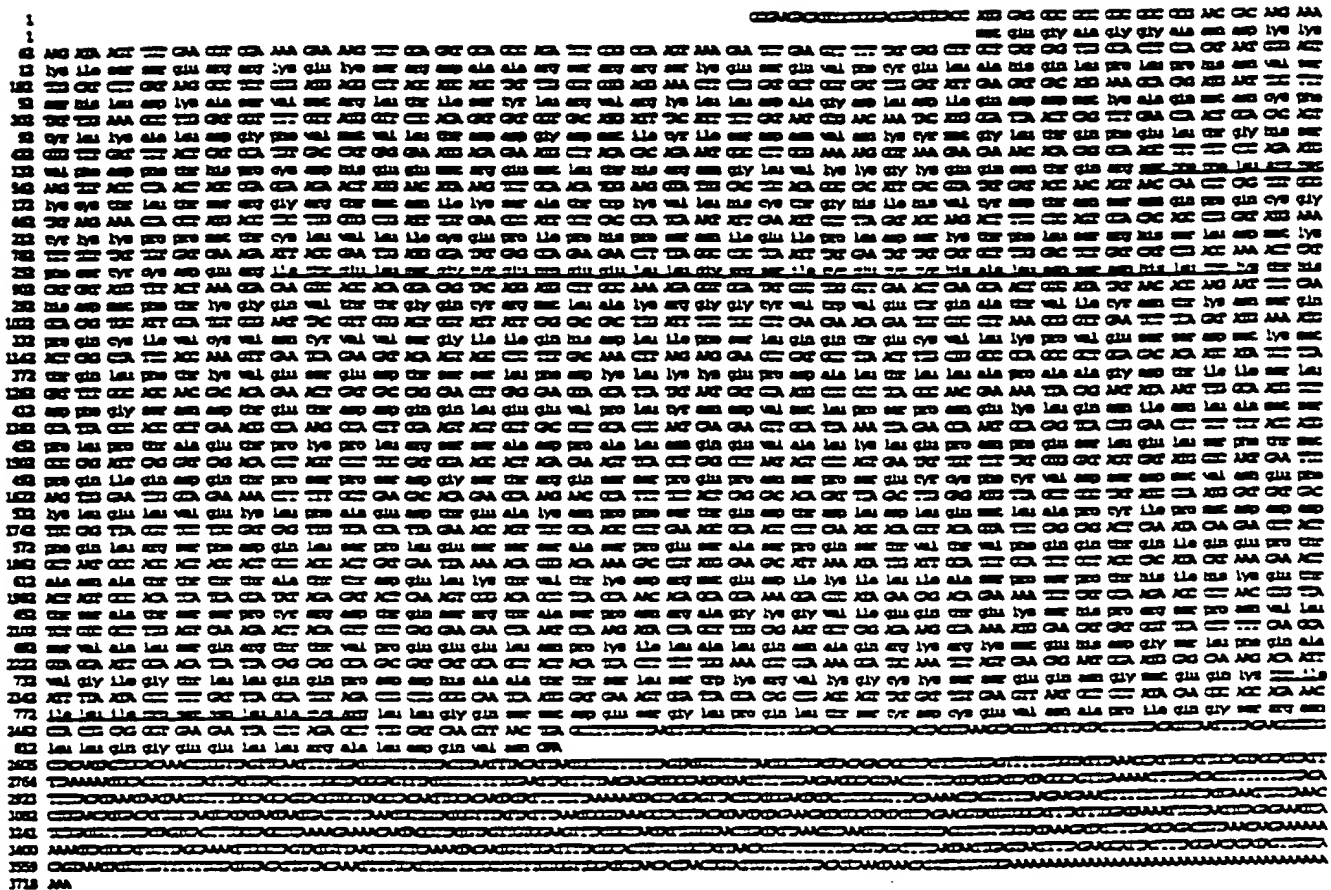


Figure 10

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**HIF-1α:** (17) RRKEKSRDAARRR SKSEVFYELAHQL PLPHNVSSILD KASVRLUTISYLRR  
**ARNT:** (90) ARENHSEI-ERRRR NKMTAYITTELSIMV PTCSALARKPD KUTILRMVSHMKSL (24%)  
**SIM:** (1) MKEKSKNAARRR EKENIEFCELAKLL PLPAATTSQLD KASVIRUTISYLRR (62%)  
**NR:** (27) AEGIKSNPS-KRIR DRINTELDRLASLL PFFQDVINKLD KLSVRLSVTYLRAK (35%)  
**CONSENSUS:** -----K3-----R-RR -K-----1-ELA-1L P1P--1---LD K-SV1RL2-SYL4-4

B A S I C      H E L I X      L O O P      H E L I X

**ARNT:** (90) ARENHSEIERRRR -NKMTAYITTELSIMV PTCSALARKPD--- KUTILRMVSHMKSL  
**MI:** (205) KKINNNLIIRRRR -FNINDRIKEIGILI PKSNPDIMRWAN-- KGHILKASVDYIRKL (32%)  
**USF:** (200) RRAQHNEVERRRR -DKINNWIVQLSKII PDCSMESTKSOQS KGGHLSKACDVIQEL (38%)  
**L-MYC:** (286) KRKNHNFLEKRRR -NDLRSRFLALRDQV PTL-ASCSKAP--- KVVTLKALEVILQAL (39%)  
**CP-1:** (223) RKDSHKEVERRRR ENINTA-INWLSDLL PVR---ESSKAA-- ----ILARAAEYTIQKL (37%)  
**CONSENSUS:** 443-HNEIERRRR ----1----I--LSD11 P-----K----- K--IL-4A13YIQ-L

Figure 11



<b>HIF-1<math>\alpha</math>:</b>	(106)	DGIMTYISINWVKYMGILQFELIGISVDFTHPCDHEEMREML--THRNGLVK	
<b>ARNT:</b>	(183)	TGRVYVSDSVTPVINQFQSEMFSGSILYDQVHPDDVDKLRQL--SISENALIT	(29%)
<b>NR:</b>	(130)	DALVFYASSITQDYILGFQQSDVTHQSVVELTHIEDRAEFQQLIWAINPSQCT	(23%)
<b>SIM:</b>	(97)	DGKIMYISETASVHILGSLQVELIGNSIFETTHNYDQDEMAAIL--SLIHHINQ	(37%)
<b>PER:</b>	(286)	DGIVLXTPTSTIDVLGYPRUMILGRSFIDFVHLKDRATFASQI--TTIGIPIAE	(22%)
<b>kinA:</b>	(24)	NGRIITYISANSKILHLYLQGMIGSFLKTFIHEEDQFLVESYFYNEHILMPCT	(26%)
<b>CONSENSUS:</b>		DG-11Y1S-21----IG--Q-E11G-S1-311H--D---1---L--2-----	
<b>HIF-1<math>\alpha</math>:</b>	(249)	DMKFSYCDERTITELMGYEPEELLGR-SIYEYTHALDSDHLLK-THIDMFTKQQ	
<b>ARNT:</b>	(370)	EGIFTVDHRCVATVGXPQQLLGLK-NIVEFCHPEDQQLLRDSFQQVVKLKGQ	(35%)
<b>NR:</b>	(288)	DFTPIGCDAKGRIVLGYTEAEELCTRGSGYQFTHAADMLYCAE-SHIRMIKTGE	(31%)
<b>SIM:</b>	(263)	DMKLIFFDARVSQITGYEPQDLIEK-TLYQYTHAADIMAMRC-SIQILLYKQQ	(39%)
<b>PER:</b>	(336)	TGLISHVDSAAVSALGYLPQDLIGR-SIMDFYHHEDLSVMKE-TYEIVMKKGQ	(33%)
<b>kinA:</b>	(288)	WFMNESGISLFEANTYE--DLIGK-NIYDQLHPCDIEDVKERIQNIAEQKTE	(19%)
<b>CONSENSUS:</b>		31-1---D-4-----1GY-P-3L1G4-2TY3--H--D---143-2---11-KGQ	

Figure 12

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Figure 13A

A. 1% OXYGEN

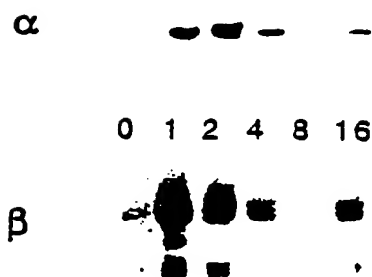


Figure 13B

B. COBALT CHLORIDE

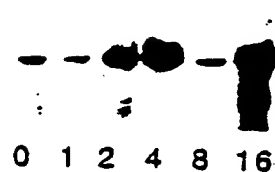


Figure 13C

C. DESFERRIOXAMINE

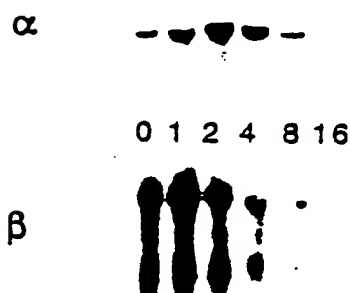


Figure 13D

D. POST-HYPOXIA



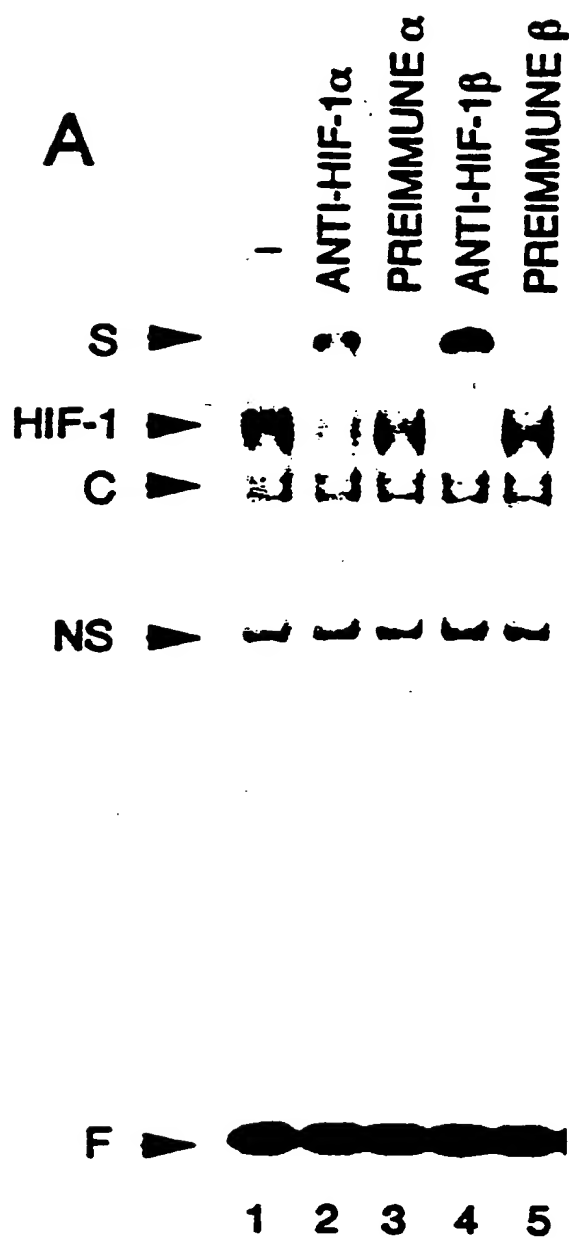
Figure 13E

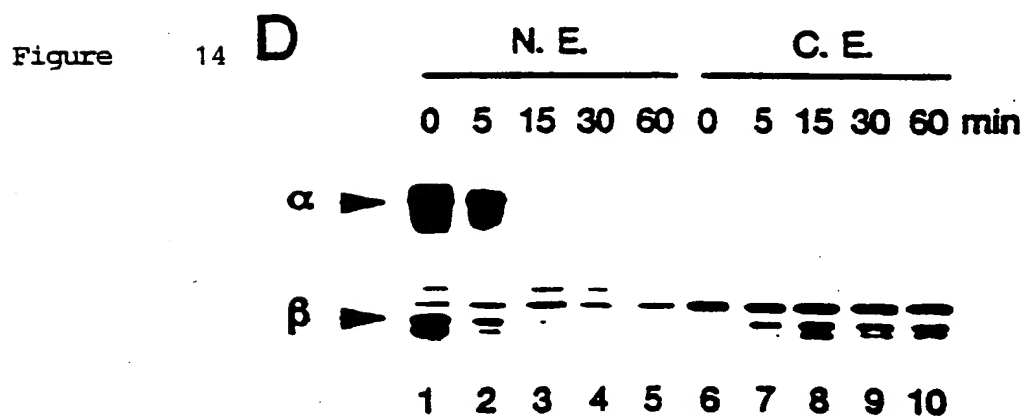
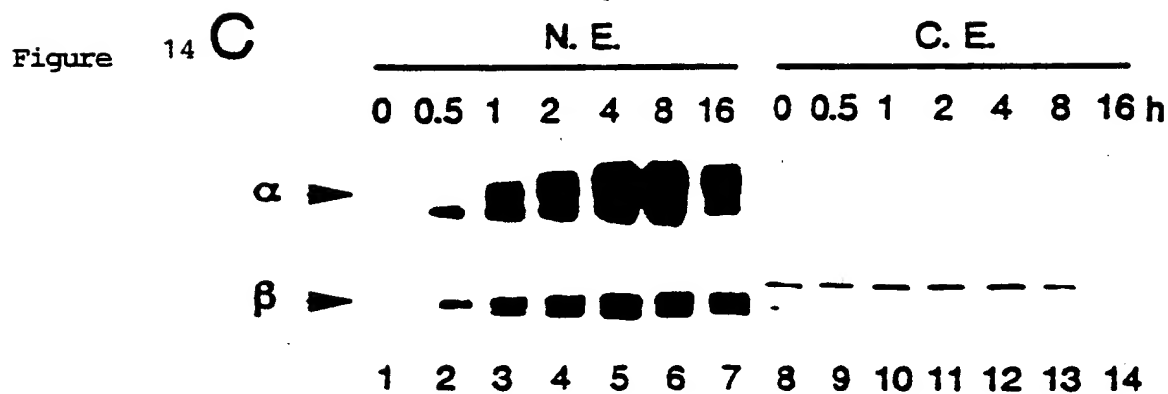
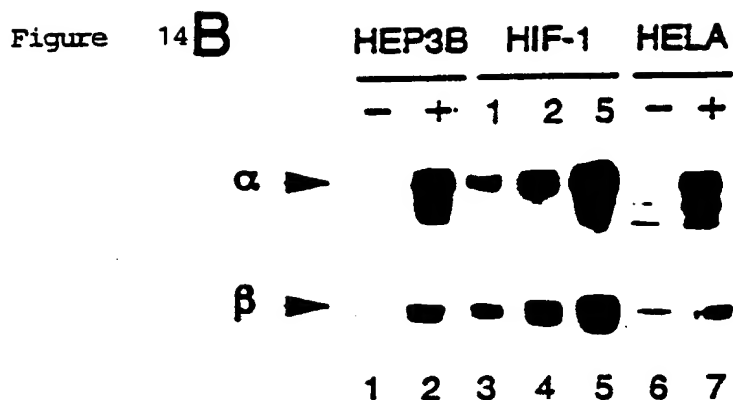
E. 3'-UNTRANSLATED SEQUENCES

2568 CU AUUUA UA  
 2656 UA AUUUA CA  
 2731 GU AUUUA AA  
 2781 UU AUUUA UU  
 2785 UU AUUUA UU  
 3138 UU AUUUA AA  
 3156 CC AUUUA CA  
 3203 GC AUUUA UU

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Figure 14A





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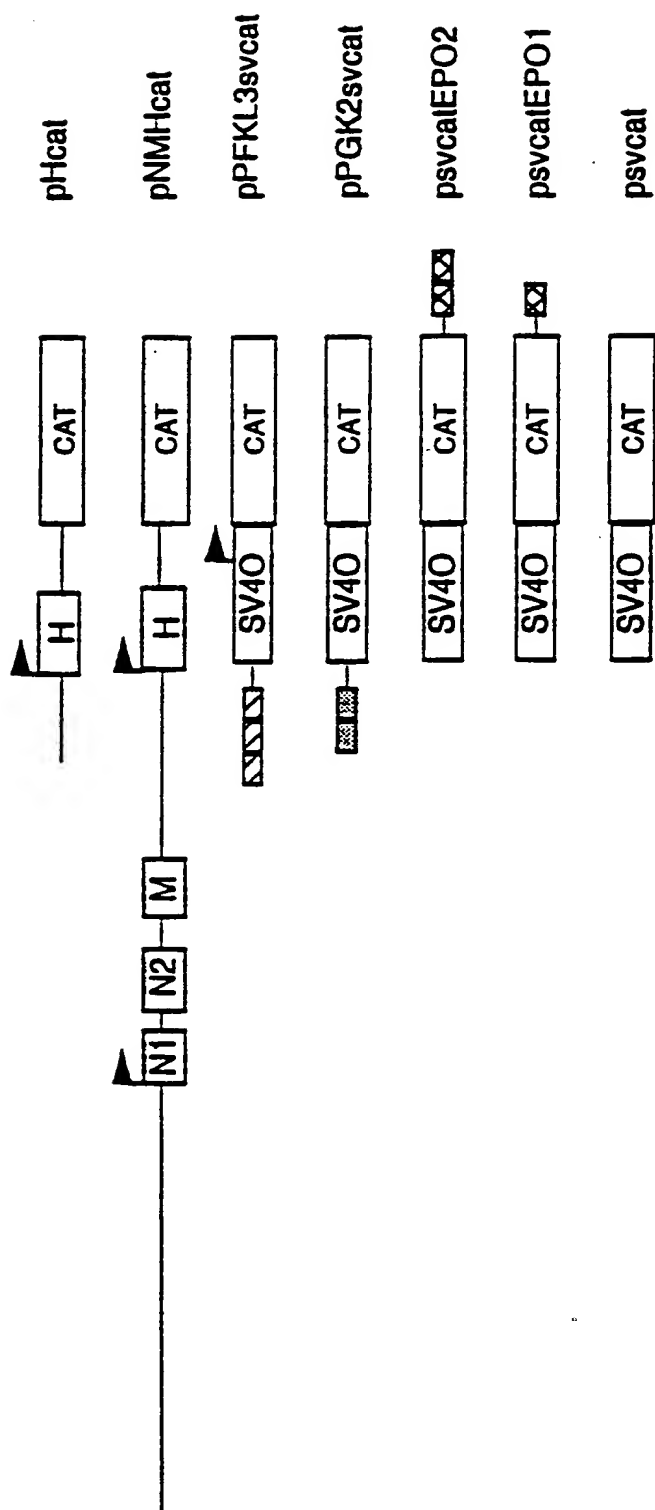


Figure 15A

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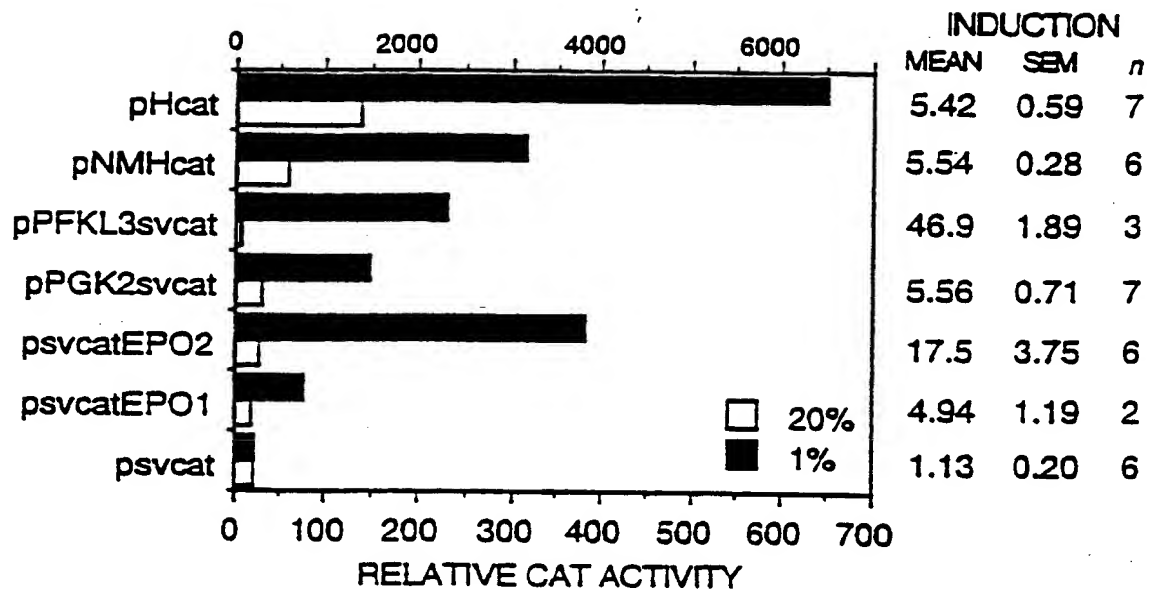


Figure 15B

Figure 16

AMINO-TERMINAL AMINO-ACID SEQUENCE (ENCOMPASSING BASIC DOMAIN)  
OF WILD-TYPE AND DOMINANT-NEGATIVE-MUTANT FORMS OF HIF-1 $\alpha$

HIF-1 $\alpha$ : 1/MEGAGGANDKKKISSERRKEKSRDAARSRR/30  
HIF-1 $\alpha$  $\Delta$ NB: 1/MEGIAG-----SRR/30  
HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB: 1/MEGIAG-----SRR/30

CARBOXY-TERMINAL AMINO-ACID SEQUENCE OF WILD-TYPE AND  
DOMINANT-NEGATIVE-MUTANT FORMS OF HIF-1 $\alpha$

HIF-1 $\alpha$ : 390/LKKEPDALT/400//820/RALDQVN/826  
HIF-1 $\alpha$  $\Delta$ NB: 390/LKKEPDALT/400//820/RALDQVN/826  
HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB: 390/LKIQT-----/395

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/10251

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/00; C07H 21/04; C12N 1/12, 1/20, 5/00, 15/63

US CL : 435/240.1, 252.1, 320.1; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 252.1, 320.1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dejgaard et al. Identification, Molecular Cloning, Expression and Chromosome Mapping of a Family of Transformation Upregulated hn RNP-K Proteins Derived by Alternative Splicing, Journal of Molecular Biology. 11 February 1994, Vol. 236, No.1, pages 33-48, especially abstract on page 33 and Figure 5 on page 40.	1-12 and 16-18
A	Benjamin et al. Activation of the Heat Shock Transcription Factor by Hypoxia in Mammalian Cells. Proceedings of the National Academy of Sciences USA. August 1990, Vol. 87, No. 16, pages 6263-6267.	1-12 and 16-18

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 AUGUST 1996	Date of mailing of the international search report 02 OCT 1996
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THANDA WAI

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/10251

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Wang et al. Hypoxia-Inducible Factor 1 is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O <sub>2</sub> Tension. Proceedings of the National Academy of Sciences USA. 06 June 1995, Vol. 92, No. 12, pages 5510-5514.	1-12 and 16-18
A, P	Norris et al. Hypoxia-Induced Protein Binding to O <sub>2</sub> -Responsive Sequences on the Tyrosine Hydroxylase Gene. The Journal of Biological Chemistry. 06 October 1995, Vol. 270, No. 40, pages 23774-23779.	1-12 and 16-18
A	Fujiwara et al. Expressed Sequence Tag (EST) GenBank Accession Numbers D56430, D53682, R71408, T32121, HS14513, T32145, T35966, M85743, R71117, T32012, and Q60265. GenBank. 30 May 1995, see sequence alignments.	3-12, 16, and 18

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/10251

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-12 and 16-18
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

MPSEARCH for SEQ ID NO:1, 2, and 4; APS, MEDLINE, BIOSIS, CAPLUS, WPIDS

search terms: hypoxia, transcription factor, mammalian, inducible

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

PCT Rule 13.1 states that the "...international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ('requirement of unity of invention')." ."

Group I, claims 1-2 and 17, drawn to polypeptides (first product).

Group II, claims 3-12, 16, and 18, drawn to polynucleotide sequences which encode an animal polypeptide, vectors encoding such sequences, and host cells containing such vectors (second product).

Group III, claims 13-15, drawn to antibodies (third product).

Group IV, claims 19 and 21, drawn to a method of using antibodies or other binding reagent (first method of using the third product).

Group V, claim 20, drawn to a method of detecting a nucleotide sequence (first method of using the second product).

Group VI, claims 22-27, drawn to a method of treatment using nucleotides (second method of using the second product).

Group VII claims 28-32, drawn to a method of treatment using antisense (third method of using the antisense of the second product).

Group VIII, claim 33, drawn to a pharmaceutical composition comprising a polypeptide (fourth product).

Group IX, claim 34, drawn to a pharmaceutical comprising a nucleotide sequence (fifth product).

Group X, claim 35, drawn to a pharmaceutical composition comprising an inhibitory nucleotide sequence (sixth product).

Unity of invention does not exist because the products of Groups I, II III, VIII, IX, and X do not share a special technical feature since they are drawn to polypeptides, nucleotide sequences, antibodies, and pharmaceutical compositions comprising a polypeptide, nucleotide sequence, or the antisense of the nucleotide sequence, which are chemically and structurally distinct. Groups IV, V, VI, and VII are drawn to different methods of using the distinct products of Group II and III.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : <b>C07K 14/00, C07H 21/04, C12N 1/12, 1/20, 5/00, 15/63</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 96/39426</b> (43) International Publication Date: 12 December 1996 (12.12.96)</p>
<p>(21) International Application Number: <b>PCT/US96/10251</b> (22) International Filing Date: <b>6 June 1996 (06.06.96)</b> (30) Priority Data: <b>08/480,473                      6 June 1995 (06.06.95)                      US</b> (71) Applicant: <b>THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).</b> (72) Inventor: <b>SEMENZA, Gregg, L.; 718 Scarlett Drive, Towson, MD 21286 (US).</b> (74) Agent: <b>HAILE, Lisa, A.; Fish &amp; Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).</b></p>		<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: <b>HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE</b></p> <p>(57) Abstract</p> <p>The purified and characterization of hypoxia-inducible factor 1 (HIF-1) is described. HIF-1 is composed of subunits HIF-1<math>\alpha</math> and HIF-1<math>\beta</math>. Purified HIF-1<math>\alpha</math> polypeptide, its amino acid sequence and polynucleotide sequence are provided. A HIF-1<math>\alpha</math> variant that dimerizes to HIF-1<math>\beta</math> producing a nonfunctional HIF-1 complex is described. Methods for the prevention and treatment of hypoxia-related disorders are provided.</p>		

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## HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE

### Statement as to Federally Sponsored Research

5 This invention was made in part with funds from the Federal government, PHS grant RO1-DK39869. The government therefore has certain rights in the invention.

### FIELD OF THE INVENTION

This invention relates to hypoxia-related proteins, and specifically to novel DNA-binding proteins which are induced by hypoxia.

### Background of the Invention

10 Mammals require molecular oxygen (O<sub>2</sub>) for essential metabolic processes including oxidative phosphorylation in which O<sub>2</sub> serves as electron acceptor during ATP formation. Systemic, local, and intracellular homeostatic responses elicited by hypoxia (the state in which O<sub>2</sub> demand exceeds supply) include erythropoiesis  
15 by individuals who are anemic or at high altitude (Jelkmann (1992) *Physiol. Rev.* 72:449-489), neovascularization in ischemic myocardium (White et al. (1992) *Circ. Res.* 71:1490-1500), and glycolysis in cells cultured at reduced O<sub>2</sub> tension (Wolfle et al. (1983) *Eur. J. Biochem.* 135:405-412). These adaptive responses either increase O<sub>2</sub> delivery or activate alternate metabolic pathways that do not require  
20 O<sub>2</sub>. Hypoxia-inducible gene products that participate in these responses include erythropoietin (EPO) (reviewed in Semenza (1994) *Hematol. Oncol. Clinics N. Amer.* 8:863-884), vascular endothelial growth factor (Shweiki et al. (1992) *Nature* 359:843-845; Banai et al. (1994) *Cardiovasc. Res.* 28:1176-1179; Goldberg & Schneider (1994) *J. Biol. Chem.* 269:4355-4359), and glycolytic enzymes (Firth et  
25 al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500; Semenza et al. (1994) *J. Biol. Chem.* 269:23757-23763).

The molecular mechanisms that mediate genetic responses to hypoxia have been extensively investigated for the EPO gene, which encodes a growth factor that regulates erythropoiesis and thus blood O<sub>2</sub>-carrying capacity (Jelkmann  
30 (1992) *supra*; Semenza (1994) *supra*). *Cis*-acting DNA sequences required for transcriptional activation in response to hypoxia were identified in the EPO 3'-flanking region and a *trans*-acting factor that binds to the enhancer,

- 2 -

hypoxia-inducible factor 1 (HIF-1), fulfilled criteria for a physiological regulator of EPO transcription: inducers of EPO expression (1% O<sub>2</sub>, cobalt chloride [CoCl<sub>2</sub>], and desferrioxamine [DFX]) also induced HIF-1 DNA binding activity with similar kinetics; inhibitors of EPO expression (actinomycin D, cycloheximide, and 2-aminopurine) blocked induction of HIF-1 activity; and mutations in the EPO 3'-flanking region that eliminated HIF-1 binding also eliminated enhancer function (Semenza (1994) supra). These results also support the hypothesis that O<sub>2</sub> tension is sensed by a hemoprotein (Goldberg et al. (1988) Science 242:1412-1415) and that a signal transduction pathway requiring ongoing transcription, translation, and protein phosphorylation participates in the induction of HIF-1 DNA-binding activity and EPO transcription in hypoxic cells (Semenza (1994) supra).

EPO expression is cell type specific, but induction of HIF-1 activity by 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX was detected in many mammalian cell lines (Wang & Semenza (1993a) Proc. Natl. Acad. Sci. USA 90:4304-4308), and the EPO enhancer directed hypoxia-inducible transcription of reporter genes transfected into non-EPO-producing cells (Wang & Semenza (1993a) supra; Maxwell et al. (1993) Proc. Natl. Acad. Sci. USA 90:2423-2427). RNAs encoding several glycolytic enzymes were induced by 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX in EPO-producing Hep3B or non-producing HeLa cells whereas cycloheximide blocked their induction and glycolytic gene sequences containing HIF-1 binding sites mediated hypoxia-inducible transcription in transfection assays (Firth et al. (1994) supra; Semenza et al. (1994) supra). These experiments support the role of HIF-1 in activating homeostatic responses to hypoxia.

## SUMMARY OF THE INVENTION

The invention features a substantially purified DNA-binding protein, hypoxia-inducible factor-1 (HIF-1), characterized as activating structural gene expression where the promoter region of the structural gene contains an HIF-1 binding site. Examples of such structural genes include erythropoietin (EPO), vascular endothelial growth hormone (V-EGF), and glycolytic genes. HIF-1 is composed of two subunits, HIF-1 $\alpha$  and an isoform of HIF-1 $\beta$ .

The invention features a substantially purified HIF-1 $\alpha$  polypeptide, and a nucleotide sequence which encodes HIF-1 $\alpha$ .



- 3 -

The invention provides methods for preventing and treating hypoxia-related disorders, including tissue damage resulting from hypoxia and reperfusion, by administering a therapeutically effective amount of HIF-1 protein. Also included in the invention is gene therapy by introducing into cells a nucleotide sequence  
5 encoding HIF-1. The invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier admixed with a therapeutically effective amount of HIF-1 or nucleotide sequence encoding HIF-1.

The invention further provides a novel HIF-1 $\alpha$  variant polypeptide which functionally inactivates HIF-1 *in vivo*. The invention provides a method for treating  
10 an HIF-1-mediated disorder or condition by functional inactivation of HIF-1 by administration of an effective amount of the HIF-1 $\alpha$  variant of the invention.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 is a autoradiograph showing dose-dependent induction of HIF-1 DNA binding activity by CoCl<sub>2</sub> treatment. Nuclear extracts, prepared from HeLa cells  
15 cultured in the presence of the 0, 5, 10, 25, 50, 75, 100, 250, 500, or 1000  $\mu$ M of CoCl<sub>2</sub> for 4 h at 37°C, were incubated with W18 probe and analyzed by gel shift assay. Lanes 1-8 and 9-12 represent extracts prepared in two separate experiments. Arrows indicate HIF-1, constitutive DNA binding activity (C), nonspecific activity (NS), and free probe (F).

Fig. 2 is an autoradiograph showing the results of methylation interference analysis with nuclear extracts from CoCl<sub>2</sub>-treated HeLa cells. W18 was 5'-end  
20 labeled on the coding or noncoding strand, partially methylated, and incubated with nuclear extracts. DNA-protein complexes corresponding to HIF-1, constitutive DNA binding activities (C1 and C2), and nonspecific binding activity (NS) were isolated from a preparative gel shift assay (lower) in addition to free  
25 probe (F) (not shown). DNA was purified, cleaved with piperidine, and analyzed on a 15% denaturing polyacrylamide gel (upper). Results are summarized at left for coding strand and at right for noncoding strand. The guanine residues are numbered according to their locations on the W18 probe. The HIF-1 binding site is boxed. Complete methylation interference with HIF-1 binding is indicated in  
30 closed circles; partial and complete methylation interference with constitutive DNA binding activity are indicated by open and closed squares, respectively.

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Fig. 3A is an autoradiograph showing gel shift assay analysis of column fractions for HIF-1 DNA binding activity. Nuclear extracts were fractionated by DEAE-Sepharose chromatography, and fractions containing HIF-1 activity were applied to a W18 DNA affinity column. 5 ug of protein were incubated with 0.1 ug of calf thymus DNA for gel shift analysis of crude nuclear extract (Crude NE, lane 1) and HIF-1 active fractions from DEAE-Sepharose columns (DEAE, lane 2). For fractions from the W18 column (lanes 3-13), 1 ul aliquots were incubated with 5 ng of calf thymus DNA. The positions of the two HIF-1 bands, constitutive activity (C), nonspecific activity (NS), and free probe (F) are indicated. FT, flowthrough, 0.25 M, 0.5 M, 1 M, and 2 M are fractions eluted with indicated concentration of KCl in buffer Z.

Fig. 3B is an autoradiograph showing sequence-specific DNA binding of the partially purified fractions described in the legend to Fig. 3A. 5 ug aliquots of fractions from the DEAE-Sepharose column were incubated with W18 probe in the presence of no competitor (lane 1), 10-fold (lanes 2 and 5), 50-fold (lanes 3 and 6), or 250-fold (lanes 4 and 7) molar excess of unlabeled W18 (W, lanes 2-4) or M18 (M, lanes 5-7) oligonucleotide.

Fig. 4A is an autoradiograph showing purification of HIF-1 from  $\text{CoCl}_2$ -treated HeLa S3 cells. Flowthrough fraction from the M18 DNA column (Load, lane 1) and 0.25 M KCl and 0.5 M KCl fractions from the second W18 DNA affinity column (lanes 2 and 3) were analyzed. An aliquot of each fraction (5 ug of load or 1 ug of affinity column fractions) were resolved by 6% SDS-PAGE and silver stained. HIF-1 polypeptides in lanes 2 and 3 are indicated by arrows at the right of the figure.

Fig. 4B is an autoradiograph showing HIF-1 purification from hypoxic Hep3B cells. HIF-1 fractions from the first W18 column (Load, lane 1) and 0.25 M KCl and 0.5 M KCl fractions from the second W18 column (lanes 2 and 3) were analyzed. An aliquot of each fraction (50 ul) was resolved by 7% SDS-PAGE and silver stained. Molecular mass markers are myosin (200 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), and ovalbumin (45 kDa). HIF-1 polypeptides in lanes 2 and 3 are indicated by arrows at the right of the figure.

- 5 -

Fig. 5A is an autoradiograph identifying the HIF-1 polypeptides. An aliquot of affinity-purified HIF-1 was resolved on a 6% SDS-polyacrylamide gel with 3.2% cross-linking along with the HIF-1 protein complex isolated by preparative native gel shift assay (HIF-1). MW, molecular mass markers with size (kDa) indicated at left of figure; numbers to the right of figure indicate the apparent molecular weights (kDa) of HIF-1 polypeptides.

Fig. 5B is an autoradiograph showing the HIF-1 components on a 6% SDS-polyacrylamide gel with 5% cross-linking. An aliquot of affinity-purified HIF-1 was resolved on a 6% SDS-polyacrylamide gel along with the HIF-1 protein complex isolated by preparative native gel shift assay (HIF-1). The 120 kDa polypeptide, 94/93/91 kDa polypeptides, and two contaminant proteins (\*1 and \*2) are indicated.

Fig. 5C is an autoradiograph showing the alignment of HIF-1 components identified on two gel systems with different degrees of cross-linking. Gel slices isolated from the 6% SDS-polyacrylamide gel with 5% cross-linking corresponding to 120 kDa HIF-1 polypeptide (12), 94/93/91 kDa HIF-1 polypeptide (94/93/91), and two contaminant proteins (\*1 and \*2) were resolved on a 6% SDS-polyacrylamide gel with 3.2% cross-linking in parallel with an aliquot (30 ul) of affinity purified HIF-1 (Fig. 5A).

Fig. 6 is a graph of the absorbance profiles at 215 nm of tryptic peptides derived from 91 kDa HIF-1 polypeptide (top), 93/94 kDa polypeptides (middle), and trypsin (bottom).

Fig. 7 is an autoradiograph showing UV cross-linking analysis with affinity purified HIF-1 and probe W18 in the absence (lane 1) or presence of 250-fold molar excess of unlabeled W18 (lane 2) or M18 (lane 3) oligonucleotide. The binding reaction mixtures were UV-irradiated and analyzed on a 6% SDS-polyacrylamide gel. Molecular mass standards are indicated at left.

Fig. 8 is an autoradiograph showing the results of glycerol gradient sedimentation analysis. Nuclear extracts prepared from Hep3B cells exposed to

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1% O<sub>2</sub> for 4 h (Load) was sedimented through a 10-30% linear glycerol gradient. Aliquots (10 ul) from each fraction were analyzed by gel shift assay. Arrows at top indicate the peak migration for ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa).

5           FIG. 9 is a diagram of the cDNA sequence encoding HIF-1 $\alpha$ . Bold lines indicate extent of clones hbc120, hbc025, and 3.2-3 relative to the full-length RNA-coding sequence shown below. Box, amino acid coding sequences; thin line, untranslated sequences; bHLH, basic helix-loop-helix domain; A and B, internal homology units within the PAS domain.

10           Fig. 10 is the nucleotide and derived amino acid sequence of HIF-1 $\alpha$ . A composite sequence was derived from the complete nucleotide sequences determined for clones 3.2-3 (nt 1-3389), hbc025 (nt 135-3691), and hbc120 (nt 1739-3720). Sequences of four tryptic peptides obtained from the purified HIF-1 $\alpha$  120 kDa polypeptide are underscored (two peptides are contiguous).

15           Fig. 11 is the analysis of bHLH domains. Coordinate of first residue of each sequence and amino acid identity with HIF- 1 $\alpha$  or HIF- 1 $\beta$  (ARNT) are given in parentheses at left and right margins, respectively. Hyphen indicates gap introduced into sequence to maximize alignment except in consensus where it indicates a lack of agreement. Consensus indicates at least 3 proteins with  
20 identical or similar residue at a given position. 1: F, I, L, M, or V; 2: S or T; 3: D or E; 4: K or R. Invariant residues are shown in bold.

Fig. 12 is the analysis of PAS domains. Alignments of PAS A (top) and B (bottom) subdomains are shown. Consensus indicates at least 4 proteins with identical or similar residue at a given position. GenBank accession numbers:  
25 ARNT, M69238; AHR, L19872; SIM, M19020; MI, Z23066; USF, X55666; L-MYC, X13945; CP-1, M34070; PER, M30114; KinA, M31067.

Fig. 13A is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 1% O<sub>2</sub> for 0, 1, 2, 4, 8, and 16 h.

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Fig. 13B is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 75  $\mu$ M CoCl<sub>2</sub> for 0, 1, 2, 4, 8, and 16 h.

Fig. 13C is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposure of Hep3B cells to 130  $\mu$ M desferrioxamine (DFX) for 0, 1, 2, 4, 8, and 16 h.

Fig. 13D is an autoradiograph showing HIF-1 $\alpha$  and HIF-1 $\beta$  RNA expression after exposing Hep3B cells to 1% O<sub>2</sub> for 4 h, then returning the cells to 20% O<sub>2</sub> for 0, 5, 15, 30, or 60 min prior to RNA isolation.

Fig. 13E is a table of the AUUUA-containing elements from the HIF-1 $\alpha$  3'-UTR. The first nucleotide is numbered according to the composite cDNA sequence.

Fig. 14A is an autoradiograph of nuclear extracts from hypoxic Hep3B cells incubated with oligonucleotide probe W18 for 10 min on ice, immune sera was added (lanes 2 and 5) and incubated for 20 min on ice, followed by polyacrylamide gel electrophoresis. Preimmune sera (lanes 3 and 5) and antisera (lanes 2 and 4) were obtained from rabbits before and after immunization, respectively, with GST/HIF-1 $\alpha$  (lanes 2 and 3) or GST/HIF-1 $\beta$  (lanes 4 and 5). HIF-1, constitutive (C) and nonspecific (NS) DNA binding activities, free probe (F), and supershifted HIF-1/DNA/antibody complex (S) are indicated.

Fig. 14B is an immunoblot showing antisera recognition of HIF-1 subunits present in purified protein preparations and crude protein extracts. Nuclear extracts from Hep3B cells which were untreated (lane 1) or exposed to 1% O<sub>2</sub> for 4 h (lane 2) and from HeLa cells which were untreated (lane 6) or exposed to 75  $\mu$ M CoCl<sub>2</sub> for 4 h (lane 7) were fractionated on a 6% SDS/polyacrylamide gel in parallel with 1, 2, and 5  $\mu$ l of affinity-purified HIF-1 from CoCl<sub>2</sub>-treated HeLa cells (lanes 3-5). Protein was transferred to a nitrocellulose membrane and incubated with antisera to HIF-1 $\alpha$  (top) or HIF-1 $\beta$  (bottom).

Fig. 14C is an immunoblot showing the induction kinetics of HIF-1 $\alpha$  and HIF-1 $\beta$  protein in hypoxic cells. Hep3B cells were exposed to 1% O<sub>2</sub> for 0 to 16 h prior to preparation of nuclear (N.E.) and cytoplasmic (C.E.) extracts, and immunoblot analysis was performed with antisera to HIF-1 $\alpha$  (top) or HIF-1 $\beta$  (bottom).

5            Fig. 14D is an immunoblot showing decay kinetics of HIF-1 $\alpha$  and HIF-1 $\beta$  polypeptides in post-hypoxic cells. Hep3B cells were exposed to 1% O<sub>2</sub> for 4 h and returned to 20% O<sub>2</sub> for 0 to 60 min prior to preparation of extracts and immunoblot analysis. Arrowheads distinguish HIF-1 subunits from cross-reacting proteins of unknown identity.

10           Fig. 15A is an diagram of the structure of reporter gene constructs used for functional analysis of HIF-1 binding sites in human aldolase A (hALDA), human phosphoglycerate kinase 1 (hPGK1), and mouse phosphofructokinase L (mPFKL) genes. Arrow, transcription initiation site; box, hEPO 3'-FS (cross-hatched), hPGK1 5'-FS (stippled), or mPFKL IVS-1 (striped) oligonucleotide (sequences are  
15 as shown in Table 3). DNA fragments from the 5'-end of the hALDA gene in pNMHcat and pHcat are 3.5 and 0.76 kb, respectively, and are colinear at the 3'-end where they are directly fused to CAT coding sequences.

Fig. 15B is a bar graph showing CAT/ $\beta$ -galactosidase expression (relative CAT activity) in transfected cells exposed to 20% O<sub>2</sub> (open bar) or 1% O<sub>2</sub> (closed  
20 bar). Data are plotted using lower scale for all results except those for pHcat, which are plotted according to the upper scale. Induction, representing the relative CAT activity at 1% O<sub>2</sub>/20%O<sub>2</sub>, was calculated for each experiment; mean and standard error of mean (SEM) were determined for results from n independent experiments.

25           Fig. 16 is the amino-terminal (top) and carboxy-terminal (bottom) amino acid sequence of the wild-type and dominant-negative variant forms of HIF-1 $\alpha$ .

#### **DETAILED DESCRIPTION OF THE INVENTION**

The invention provides a substantially pure hypoxia-inducible factor-1 (HIF-1) characterized as a DNA-binding protein which binds to a region in the regulatory,  
30 preferably in the enhancer region, of a structural gene having the HIF-1 binding

motif. Included among the structural genes which can be activated by HIF-1 are erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic gene transcription in cells subjected to hypoxia. Analysis of purified HIF-1 shows that it is composed of subunits HIF-1 $\alpha$  and an isoform of HIF-1 $\beta$ . In addition to  
5 having domains which allow for their mutual association in forming HIF-1, the  $\alpha$  and  $\beta$  subunits of HIF-1 both contain DNA-binding domains. The alpha subunit is uniquely present in HIF-1, whereas the beta subunit (ARNT) is a component of at least two other transcription factors.

The invention provides a substantially pure hypoxia-inducible factor-1 $\alpha$  (HIF-  
10 1 $\alpha$ ) polypeptide characterized as having a molecular weight of 120 kDa as determined by SDS-PAGE and having essentially the amino acid sequence of SEQ ID NO:2 (Fig. 10) and dimerizing to HIF-1 $\beta$  to form HIF-1. The term "substantially pure" as used herein refers to HIF-1 $\alpha$  which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally  
15 associated. One skilled in the art can purify HIF-1 $\alpha$  using standard techniques for protein purification. The substantially pure polypeptide will yield a single band on a non-reducing polyacrylamide gel. The purity of the HIF-1 $\alpha$  polypeptide can also be determined by amino-terminal amino acid sequence analysis. HIF-1 $\alpha$  protein includes functional fragments of the polypeptide, as long as the activity of HIF-1 $\alpha$ ,  
20 such as the ability to bind with HIF-1 $\beta$ , remains. Smaller peptides containing the biological activity of HIF-1 $\alpha$  are included in the invention.

The invention provides nucleotide sequences encoding the HIF-1 $\alpha$  polypeptide (SEQ ID NO:1)(Fig. 10). These nucleotides include DNA, cDNA, and RNA sequences which encode HIF-1 $\alpha$ . It is also understood that all nucleotide  
25 sequences encoding all or a portion of HIF-1 $\alpha$  are also included herein, as long as they encode a polypeptide with HIF-1 $\alpha$  activity. Such nucleotide sequences include naturally occurring, synthetic, and intentionally manipulated nucleotide sequences. For example, HIF-1 $\alpha$  nucleotide sequences may be subjected to site-directed mutagenesis. The nucleotide sequence for HIF-1 $\alpha$  also includes  
30 antisense sequences. The nucleotide sequences of the invention include sequences that are degenerate as a result of the genetic code. All degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of HIF-1 $\alpha$  polypeptide which is encoded by the nucleotide sequence is functionally unchanged.

Specifically disclosed herein is a DNA sequence encoding the human HIF-1 $\alpha$  gene. The sequence contains an open reading frame encoding a polypeptide 826 amino acids in length. The human HIF-1 $\alpha$  initiation methionine codon shown in FIG. 10 at nucleotide position 29-31 is the first ATG codon following the in-frame stop codon at nucleotides 2-4. Preferably, the human HIF-1 $\alpha$  amino acid sequence is SEQ ID NO:2.

The nucleotide sequence encoding HIF-1 $\alpha$  includes SEQ ID NO:1 as well as nucleic acid sequences complementary to SEQ ID NO:1. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:2 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-identified nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA or RNA that encodes the polypeptide of SEQ ID NO:2 under physiological conditions. Specifically, the fragments should hybridize to DNA or RNA encoding HIF-1 $\alpha$  protein under stringent conditions.

Minor modifications of the HIF-1 $\alpha$  primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the HIF-1 $\alpha$  polypeptide described herein. Such proteins include those as defined by the term "having essentially the amino acid sequence of SEQ ID NO:2". Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of HIF-1 $\alpha$  still exists. Further, deletions of one or more amino acids can also result in modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which are not required for HIF-1 $\alpha$  biological activity.

The HIF-1 $\alpha$  polypeptide of the invention encoded by the nucleotide sequence of the invention includes the disclosed sequence (SEQ ID NO:2) and conservative variations thereof. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, or methionine for another, or the



substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

The DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization techniques which are well known in the art. These include, but are not limited to: 1) hybridization of genomic or cDNA libraries with probes to detect homologous nucleotide sequences, 2) polymerase-chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to the DNA sequence of interest, and 3) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features.

Preferably the HIF-1 $\alpha$  nucleotide sequence of the invention is derived from a mammalian organism, and most preferably from human. Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequences must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Sambrook et al. (1989) Molecular

Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY).

5       The development of specific DNA sequences encoding HIF-1 $\alpha$  can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of the three  
10       above-noted methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

15       The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of  
20       plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that express the gene of interest at a high level. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide  
25       are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay et al. (1983) Nucl. Acid Res., 11:2325).

30       A cDNA expression library, such as lambda gt11, can be screened indirectly for HIF-1 $\alpha$  peptides having at least one epitope, using antibodies specific for HIF-1 $\alpha$ . Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of HIF-1 $\alpha$  cDNA.

DNA sequences encoding HIF-1 $\alpha$  can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the HIF-1 $\alpha$  nucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the HIF-1 $\alpha$  genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription in the host of the inserted genetic sequence. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al. (1987) Gene 56:125), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans (1988) J. Biol. Chem. 263:3521) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedron promoters).

Nucleotide sequences encoding HIF-1 $\alpha$  can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and

subsequently treated by the  $\text{CaCl}_2$  method using procedures well known in the art. Alternatively,  $\text{MgCl}_2$  or  $\text{RbCl}$  can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

5 When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the HIF-1 $\alpha$  of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine  
10 kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see, for example, Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

15 Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

The HIF-1 $\alpha$  polypeptides of the invention can also be used to produce antibodies which are immunoreactive or bind to epitopes of the HIF-1 $\alpha$   
20 polypeptides. Such antibodies can be used, for example, in standard affinity purification techniques to isolate HIF-1 $\alpha$  or HIF-1. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods  
25 well known in the art (Kohler et al. (1975) Nature 256:495; Current Protocols in Molecular Biology, Ausubel et al., ed., 1989).

For purposes of the invention, an antibody or nucleic acid probe specific for HIF-1 $\alpha$  may be used to detect HIF-1 $\alpha$  polypeptide (using antibody) or nucleotide sequences (using nucleic acid probe) in biological fluids or tissues. The antibody  
30 reactive with HIF-1 $\alpha$  or the nucleic acid probe is preferably labeled with a compound which allows detection of binding to HIF-1 $\alpha$ . Any specimen containing a detectable amount of antigen or polynucleotide can be used. Various detectable labels and assay formats are well known to those of ordinary skill in the art and can be utilized without resort to undue experimentation.

When the cell component is nucleic acid, it may be necessary to amplify the nucleic acid prior to binding with an HIF-1 $\alpha$  specific probe. Preferably, polymerase chain reaction (PCR) is used, however, other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The present invention provides a HIF-1 $\alpha$  variant polypeptide characterized as dimerizing with HIF-1 $\beta$  to form a functionally inactive HIF-1 complex in that the complex is not able to sufficiently bind to the HIF-1 binding motif in the regulatory region to allow efficient expression of the structural gene under control of the regulatory region. The invention further provides nucleotide sequences encoding HIF-1 $\alpha$  variants. In one specific embodiment, the polynucleotide encoding HIF-1 $\alpha$  variant is provided having the polynucleotide sequence of SEQ ID NO:3. The HIF-1 $\alpha$  variant polypeptide SEQ ID NO:4 is generated by substitution of wild-type amino acids with different amino acids and by deleting a portion of the wild-type sequence. Modifications of the HIF-1 $\alpha$  variant amino acid sequence are encompassed by the invention so long as the resulting polypeptide dimerizes to HIF-1 $\beta$  to form a functionally inactive HIF-1 complex in the sense that the HIF-1 complex or dimer no longer sufficiently binds DNA. In a preferred embodiment of the invention, specific HIF-1 $\alpha$  variants are provided wherein one or more the amino acids that participate in the binding of HIF-1 to DNA are replaced using techniques of genetic engineering.

The specific dominant-negative variant forms of HIF-1 $\alpha$  are HIF-1 $\alpha$  $\Delta$ NB and HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB (see Example 10). These two forms have in common a deletion of the amino acids that comprise the basic domain required for DNA binding (HIF-1 $\alpha$  amino acid residues 17-30; Fig. 10). Any variant form of HIF-1 $\alpha$  in which modification of the basic domain eliminates DNA binding activity while maintaining the ability of HIF-1 $\alpha$  to dimerize with HIF-1 $\beta$  should function as a dominant negative variant. Such alterations of the nucleotide sequence encoding the basic domain include deletions or substitutions of critical basic amino acid residues within the domain that are required for DNA binding. Additional modifications of the protein may enhance the dominant negative effect *in vivo*. For example, the HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB variant contains the same mutation in the basic domain as HIF-1 $\alpha$  $\Delta$ NB (Fig. 16) but, in addition, HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB is also truncated at the carboxy terminus to improve its protein stability *in vivo*.

The nucleotide sequences encoding HIF-1 $\alpha$  variant molecules of the invention can be inserted into an appropriate expression vector and expressed in cells. Modified versions of the specific HIF-1 $\alpha$  variant of SEQ ID NO:4 can be engineered to enhance stability, production, purification, or yield of the expressed product. For example, the expression of a fusion protein or a cleavable fusion protein comprising the HIF-1 $\alpha$  variant and a heterologous protein can be engineered. Such a fusion protein can be readily isolated by affinity chromatography, e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the HIF-1 $\alpha$  moiety and the heterologous protein, the HIF-1 $\alpha$  polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that disrupts the cleavage site (Booth et al. (1988) *Immunol. Lett.* 19:65-708; Gardella et al. (1990) *J. Biol. Chem.* 265:15854-15859).

The invention provides methods for treatment of HIF-1-mediated disorders, including hypoxia-mediated tissue damage, which are improved or ameliorated by modulation of HIF-1 gene expression or activity. The term "modulate" envisions the inhibition of expression of HIF-1 when desirable, or enhancement of HIF-1 expression when appropriate. Where expression or enhancement of expression of HIF-1 is desirable, the method of the treatment includes direct (protein) or indirect (nucleotide) administration of HIF-1.

According to the method of the invention, substantially purified HIF-1 or the nucleotide sequence encoding HIF-1 is introduced into a human patient for the treatment or prevention of HIF-1-mediated disorders. The appropriate human patient is a subject suffering from a HIF-1-mediated disorder or a hypoxia-related disorder, such as atherosclerotic coronary or cerebral artery disease. When a patient is treated with nucleotide, the nucleotide can be a sequence which encodes HIF-1 $\alpha$  or a nucleotide sequence which encodes HIF-1 $\alpha$  and a nucleotide sequence which encodes HIF-1 $\beta$  (see, for example, Rayes, *et al.*, *Science*, 256:1193-1195, 1992; and Hoffman, *et al.*, *Science*, 252:954-958, 1991).

Where inhibition of HIF-1 $\alpha$  expression is desirable, such as the inhibition of tumor proliferation mediated by VEGF-induced angiogenesis, inhibitory nucleic acid sequences that interfere with HIF-1 expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes,

or triplex agents to block transcription or translation of a specific HIF-1 $\alpha$  mRNA or DNA, either by masking that mRNA with an antisense nucleic acid or DNA with a triplex agent, or by cleaving the nucleotide sequence with a ribozyme.

5 Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub (1990) Scientific American 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15  
10 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target HIF-1 $\alpha$ -producing cell.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix.  
15 Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher et al. (1991) Antisense Res. and Dev. 1:227; Helene (1991) Anticancer Drug Design, 6:569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA in a manner analogous to DNA restriction  
20 endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech (1988) J. Amer. Med. Assn. 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

25 There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff (1988) Nature 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11 - 18 bases in length. The longer the recognition sequence, the greater the likelihood that the  
30 sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

Suppression of HIF-1 function can also be achieved through administration of HIF-1 $\alpha$  variant polypeptide (dominant negative variant form), or a nucleotide sequence encoding HIF-1 $\alpha$  variant polypeptide. For example, in the case of disorders enhanced by expression of HIF-1 $\alpha$ , such as tumor proliferation  
5 secondary to VEGF-mediated angiogenesis, it would be desirable to "starve" the tumor by inhibiting neovascularization necessary to supply sufficient nutrients to the tumor. By administering HIF-1 $\alpha$  variant polypeptide or a nucleotide sequence encoding such polypeptide, the variant will compete with wild-type HIF-1 $\alpha$  for binding to HIF-1 $\beta$  in forming HIF-1 dimer thereby lowering the concentration of  
10 HIF-1 dimer in the cell which can efficiently bind to the HIF-1 DNA binding motif.

The present invention also provides gene therapy for the treatment of hypoxia-related disorders, which are improved or ameliorated by the HIF-1 polypeptide. Such therapy would achieve its therapeutic effect by introduction of the HIF-1 $\alpha$  nucleotide, alone or in combination with HIF-1 $\beta$  nucleotide, into cells  
15 exposed to hypoxic conditions. Delivery of HIF-1 $\alpha$  nucleotide, alone or in combination with HIF- $\beta$  nucleotide, can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Especially preferred for therapeutic delivery of sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, adeno-associated virus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine  
20 leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). Preferably, when the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a  
25 selectable marker so that transduced cells can be identified and generated. By inserting a HIF-1 $\alpha$  sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred  
30



targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the HIF-1 $\alpha$  nucleotide sequence.

5 Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to  $\Psi$ 2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

15 Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag*, *pol* and *env*, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

20 Another targeted delivery system for HIF-1 $\alpha$  nucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LW), which range in size from 0.2-4.0  $\mu$ m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al. (1981) Trends Biochem. Sci. 6:77). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene

transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al. (1988) *Biotechniques* 6:682).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with sterols, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidyl-glycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

Due to the biological activity of HIF-1 in enhancing synthesis of VEGF, EPO, and glycolytic enzymes, there are a variety of applications using the polypeptide or nucleotide of the invention. Such applications include treatment of hypoxia-related tissue damage and HIF-1-mediated disorders. In addition, HIF-1 may be useful in various gene therapy procedures. HIF-1 can be used to prevent or repair hypoxia-mediated tissue damage. Important applications include the treatment of cerebral and coronary artery disease.

Conversely, blocking HIF-1 action either with anti-HIF-1 antibodies, anti-HIF-1 $\alpha$  antibodies, or with an HIF-1 $\alpha$  antisense nucleotide might slow or ameliorate diseases dependent on HIF-1 action, e.g., V-EGF-promoted tumor vascularization. The above described method for delivering an HIF-1 $\alpha$  nucleotide are fully applicable to delivery of an HIF-1 antagonist for specific blocking of HIF-1 expression and/or activity when desirable. An HIF-1 antagonist can be an HIF-1 antibody, an HIF-1 $\alpha$  antibody, an HIF-1 $\alpha$  antisense nucleotide sequence, or the polypeptide or nucleotide of an HIF-1 $\alpha$  variant.

The isolation and purification of HIF-1 from EPO-producing Hep3B cells and non-EPO-producing HeLa S3 cells is described in Examples 1-3. HIF-1 protein was purified 11,250-fold by DEAE ion-exchange and DNA affinity chromatography. Analysis of HIF-1 revealed 4 polypeptides having molecular weights of 91, 93, 94 (HIF-1 $\beta$ ) and 120 kDa (HIF-1 $\alpha$ ). Glycerol gradient sedimentation analysis indicates that HIF-1 exists predominantly as a heterodimer and to a lesser extent as a heterotetramer.

The HIF-1 $\alpha$  polypeptide was isolated and sequenced. Its cDNA was generated by PCR and its sequence determined. The HIF-1 $\alpha$  polypeptide is characterized as a basic-helix-loop-helix (bHLH) polypeptide containing a PAS domain whose expression is regulated by cellular O<sub>2</sub> tension (Examples 4-7).

Induction of the transcription of genes encoding the glycolytic enzymes by HIF-1 was investigated (Example 9). The studies revealed that the glycolytic enzymes aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), and pyruvate kinase M (PKM) are induced by exposure of cells to HIF-1 inducers (1% O<sub>2</sub>, CoCl<sub>2</sub>, DFX). These genes have HIF-1 binding sites which were shown to specifically bind HIF-1. These results support the role of HIF-1 as a mediator of adaptive responses to hypoxia that underlie cellular and systemic oxygen homeostasis.

A dominant-negative variant of HIF-1 $\alpha$  was generated lacking the basic domain (amino acid 17-30) of the protein which is required for the binding of HIF-1 to DNA (Example 10). The variant HIF-1 $\alpha$  subunit can dimerize with HIF-1 $\beta$ , but the resulting heterodimer cannot bind DNA. In cells overexpressing the variant  
5 HIF-1 $\alpha$  subunit, the majority of the HIF-1 $\beta$  subunits were engaged in non-functional heterodimers, resulting in functional inactivation of HIF-1. These results show that the HIF-1 $\alpha$  variant is useful *in vivo* for blocking HIF-1 activity.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to  
10 those skilled in the art may alternatively be used.

**Example 1.      Experimental Methods.**

Human HIF-1 was purified, and its DNA binding activity characterized as follows.

Cell Culture and Nuclear Extract Preparation.      Human Hep3B and HeLa  
15 cells were maintained and treated with 1% O<sub>2</sub> and CoCl<sub>2</sub> (Wang & Semenza (1993a) Proc. Natl. Acad. Sci. USA 90:4304-4308), and nuclear extracts were prepared as described previously (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454; Dignam et al. (1983) Nucleic Acids Res. 11:1474-1489). HeLa S3 cells, obtained from American Type Culture Collection were adapted to  
20 suspension growth in Spinner's minimum essential medium supplemented with 5% (v/v) horse serum (Quality Biological, Gaithersburg, MD). The cells were grown to a density of 8 x 10<sup>5</sup> cells/ml and maintained by dilution to 2 x 10<sup>5</sup> cells/ml with fresh complete medium every 2 days. For induction of HIF-1 DNA binding activity, HeLa S3 cells were treated with 125  $\mu$ M CoCl<sub>2</sub> for 4 h at 37 °C before  
25 harvesting by centrifugation for 10 min at 2,500 x g. Cell pellets were washed twice with ice cold phosphate-buffered saline and resuspended in 5 packed cell volumes of buffer A (10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl) supplemented with 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride and 1 mM Na<sub>3</sub>VO<sub>4</sub>. After incubation on ice for 10 min, cells were pelleted  
30 at 2,500 x g for 5 min, resuspended in 2 packed cell volumes of buffer A, and lysed by 20 strokes in a glass Dounce homogenizer with type B pestle. Nuclei were pelleted at 10,000 x g for 10 min and resuspended in 3.5 packed nuclear

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volumes of buffer C (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM  $\text{MgCl}_2$ ) supplemented with 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mM  $\text{Na}_3\text{VO}_4$ . Nuclear proteins were extracted by stirring at 4°C for 30 min. After centrifugation at 15,000 x g for 30 min, the supernatant was dialyzed against  
5 buffer Z-100 (25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , and 100 mM KCl) at 4°C. The dialysate was clarified by ultracentrifugation at 100,000 x g for 60 min at 4°C, and designated as crude nuclear extract. The nuclear extracts were aliquoted, frozen in liquid  $\text{N}_2$ , and stored at -80°C. Protein concentration was determined by  
10 the method of Bradford (1976) Anal. Biochem. 72:248-254, with a commercial kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Gel shift assays. Gel shift assays were performed as described (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454, herein specifically incorporated by reference) except that the binding reaction was in buffer Z-100. For gel shift  
15 assays with partially purified and affinity-purified HIF-1 preparations, 0.25 mg/ml of BSA and 0.05% Nonidet P-40 were included in the binding reaction. Nonspecific competitor calf thymus DNA (Sigma) was used in reduced amounts for partially purified fractions, and no calf thymus DNA was used for affinity-purified HIF-1 fractions. For competition experiments, unlabeled oligonucleotide  
20 DNA was incubated with DEAE-Sepharose column fractions for 5 min on ice before probe DNA was added.

Nuclear extracts prepared from HeLa cells cultured in the presence of 0, 5, 10, 25, 50, 75, 100, 250, 500 or 1000  $\mu\text{M}$   $\text{CoCl}_2$  for 4 h at 37°C, were incubated with W18 probe.

25 Methylation interference analysis. Methylation interference analysis was performed as described (Wang & Semenza (1993b) J. Biol. Chem. 268:21513-21518, herein specifically incorporated by reference), except 100  $\mu\text{g}$  of nuclear extract prepared from  $\text{CoCl}_2$ -treated HeLa cells were used in the binding reactions.

30 Results. To determine the optimal concentration of  $\text{CoCl}_2$  for induction of HIF-1 DNA binding activity, HeLa cells were treated with  $\text{CoCl}_2$ . Nuclear extracts were prepared and analyzed by gel shift assay with the wild-type oligonucleotide W18 (Example 2) as probe. Results are shown in Fig. 1. Induction of HIF-1 DNA binding activity by  $\text{CoCl}_2$  was dose-dependent. HIF-1 activity in nuclear extracts

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was detected at 25  $\mu\text{M}$   $\text{CoCl}_2$  and reached a peak activity at 250  $\mu\text{M}$ . Significant cell death, however, was observed at  $\text{CoCl}_2$  concentrations greater than 250  $\mu\text{M}$ , resulting in decreased yield of nuclear proteins. For this reason 125  $\mu\text{M}$   $\text{CoCl}_2$  was chosen for subsequent large scale nuclear extract preparation. Constitutive DNA binding activities, which also bind W18 probe sequence specifically remained relatively unchanged in cells treated with 0-100  $\mu\text{M}$   $\text{CoCl}_2$ , and decreased at  $\text{CoCl}_2$  concentration greater than 250  $\mu\text{M}$ , suggesting an adverse effect of high  $\text{CoCl}_2$  concentration on the cells. Nonspecific DNA binding activities were barely detectable in this particular gel shift assay and vary with cell type and the relative amount of nonspecific competitor DNA used.

Methylation interference analysis was performed to determine if HIF-1 from hypoxic Hep3B cells and  $\text{CoCl}_2$ -treated HeLa cells has the same DNA binding properties. As shown in Fig. 2, methylation of  $G_8$  or  $G_{10}$  on the coding strand eliminated or greatly reduced HIF-1 binding, respectively (Fig. 2, left, lane 2). Methylation of  $G_{10}$  only partially interfered with the binding of constitutive factors (Fig. 2, left, lanes 3 and 4). On the noncoding strand, methylation of  $G_7$  or  $G_{11}$  blocked HIF-1 binding to the probe (Fig. 2B, right, lane 2). Only the methylation of  $G_7$  interfered with binding of constitutive factors (Fig. 2B, right, lanes 3 and 4). The nonspecific binding activity was unaffected by DNA methylation on either strand (Fig. 2A, left, lane 5 and Fig. 2B, right, lane 5). The results indicate that (i) HIF-1 closely contacts  $G_8$  and  $G_{10}$  on the coding strand and  $G_7$  and  $G_{11}$  on the noncoding strand through the major groove of the DNA helix, and (ii) HIF-1 and the constitutive DNA binding factors can be distinguished by the nature of their DNA binding site contacts.

**Example 2.      Biochemical Purification of HIF-1.**

Preparation of DNA affinity columns. DNA affinity columns were prepared by coupling multimerized double-stranded oligonucleotides to CNBr-activated Sepharose (Kadonaga & Tijan (1986) Proc. Natl. Acad. Sci. USA 83:5889-5893).

5      The wild-type and the mutant column contained multimerized oligonucleotide W18 (SEQ ID NO:5) and M18 (SEQ ID NO:6) (mutation underlined), respectively.

W18:      5'-gatcGCCCTACGTGCTGTCTCA-3'  
            3'-CGGGATGCACGACAGAGTctag-5'

10      M18:      5'-gatcGCCCTAAAAGCTGTCTCA-3'  
            3'-CGGGATTTTCGACAGAGTctag-5'

Equal amounts of complementary oligonucleotides were annealed, phosphorylated, and ligated. Ligated oligonucleotides (60-500 bp) were extracted with phenol/chloroform, ethanol precipitated, resuspended in deionized water, and  
15      coupled to CNBr-activated Sepharose 4B as instructed by the manufacturer (Pharmacia Biotech Inc.). Approximately 50 ug of ligated double-stranded oligonucleotides were coupled per ml of Sepharose.

Purification of HIF-1. Crude nuclear extracts from 120 liters of CoCl<sub>2</sub>-treated HeLa S3 cells (435 ml, 3,040 mg) were thawed on ice and clarified by  
20      centrifugation at 15,000 x g for 10 min. Extracts were fractionated as three batches over a 36 ml DEAE-Sepharose CL-6B column (Pharmacia) in buffer Z-100 with a step gradient of increasing KCl. Fractions containing peak activity were pooled and dialyzed against buffer Z-100. The dialysate from DEAE-Sepharose columns was incubated with calf thymus DNA (Sigma) at a  
25      concentration of 4.4 ug/ml for 15 min on ice. After centrifugation at 15,000 x g for 10 min, the supernatant (240 ml; 2.3 mg/ml) was applied to a 6 ml DNA affinity column prepared with concatenated W18 oligonucleotide. The fractions containing HIF-1 activity were pooled and dialyzed against buffer Z-100. The dialysate from the first DNA-affinity column was mixed with calf thymus DNA at a  
30      concentration of 2.5 ug/ml and incubated on ice for 15 min. After centrifugation (as described above), the supernatant was applied to a 1.5 ml M18 DNA-Sepharose column. The flowthrough from the M18 column was collected and

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reapplied to a second 2 ml W18 column. All buffers used for DNA affinity chromatography were supplemented with 0.05% Nonidet P-40 and 5 mM DTT. The amount of protein in affinity column fractions was quantitated by silver staining of SDS-polyacrylamide gels or by Amido Black (Sigma) staining of nitrocellulose membranes (Schleicher & Schuell) spotted with protein samples and compared against known amounts of proteins standards (Bio-Rad).

For purification of HIF-1 from hypoxia-treated Hep3B cells, nuclear extracts (95 mg) were fractionated by the use of a 4 ml DEAE-Sepharose CL-6B column as described above. 0.25 M KCl elute fractions were dialyzed against buffer Z-100 and applied onto a Sephacryl S-300 gel filtration column (50 ml, 1.5 x 30 cm). The fractions containing HIF-1 activity were pooled and applied to a 2 ml calf thymus DNA column (0.8 mg of calf thymus DNA/ml of Sepharose) prepared by coupling single-stranded calf thymus DNA to CNBr-activated Sepharose 4B. The flowthrough was collected and applied to a 0.4 ml W18 column as described above after incubation with calf thymus DNA (2.2 ug/ml) for 10 min followed by another 0.2 ml W18 column after dialysis against buffer Z-100.

SDS-PAGE and Silver Staining. SDS-PAGE was carried out as described by Laemmli (1970) Nature 227:680-685. The gels were calibrated with high range molecular weight standards or prestained molecular weight markers (Bio-Rad). Electrophoresis was performed at 30 mA. Silver staining was performed with silver nitrate as described (Switzer et al. (1979) Anal. Biochem. 98:231-237). Molecular weight estimation for HIF-1 polypeptides was based on SDS-polyacrylamide gels with 3.2% cross-linking (acrylamide/bisacrylamide ration of 30:1).

Results. Since HIF-1 DNA binding activity from hypoxic Hep3B cells and CoCl<sub>2</sub>-treated HeLa cells are indistinguishable (Example 1), HeLa S3 cells treated with 125 uM CoCl<sub>2</sub> were used as starting material for the large scale purification of HIF-1. To purify HIF-1 by DNA affinity chromatography, the constitutive DNA binding activity had to first be separated from HIF-1 since both bind specifically to the W18 DNA sequence. Various ion-exchange resins and gel filtration matrices were examined. HIF-1 was retained on DEAE anion-change resins in buffer Z-100, whereas constitutive DNA binding activity was found in the flowthrough. HIF-1 DNA binding activity was eluted with 250 mM KCl in buffer Z. DEAE-Sepharose chromatography effectively removed constitutive DNA binding



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activity and resulted in a 4-fold purification of HIF-1 (Fig. 3A, lanes 1 and 2). This step, however, appeared to destabilize the HIF-1 protein complex and resulted in a faster migrating form of HIF-1 (Fig. 3A, lane 2, second arrow), which was also occasionally seen in crude nuclear extract preparations. This faster migrating form could be converted to the slower migrating HIF-1 band at higher salt concentrations, and HIF-1 appeared predominantly as the slower migrating form again after the first round of DNA affinity column chromatography (Fig. 3A, lanes 10-12), suggesting that no HIF-1 component was lost during the DEAE-Sephacrose chromatography step. Probe binding of both HIF-1 forms could be competed by unlabeled W18 (Fig. 3B, lanes 2-4) but not M18 oligonucleotide (Fig. 3B, lanes 5-7), which contained a three-base pair substitution that abolished the ability of the EPO enhancer to mediate hypoxia-inducible transcription.

Partially purified HIF-1 fractions were then incubated with nonspecific competitor calf thymus DNA at concentrations that allowed optimal detection of HIF-1 DNA binding activity by gel shift assays and applied to a W18 DNA affinity column. Eluted fractions containing HIF-1 (0.5 M KCl, Fig. 3A, lane 10; 1 M KCl, Fig. 3A, lane 11) were pooled and dialyzed against buffer Z-100. To eliminate nonspecific DNA-binding proteins that were not removed by calf thymus DNA competitor, the dialysate was applied to an M18 DNA column. HIF-1 DNA binding activity was detected in the flowthrough, which was then applied directly onto second W18 column. HIF-1 activity was detected exclusively in 0.5 M KCl fractions. Two rounds of W18 and one round of M18 column chromatography resulted in a purification of approximately 2,800-fold.

The results of the final large scale purification are summarized in Table 1. From 120 liters of HeLa cells, approximately 60  $\mu$ g of highly purified HIF-1 were obtained. The total purification was 11,250-fold and yielded approximately 22% of the starting of HIF-1 DNA binding activity. Our objective was to identify HIF-1 subunits and isolate HIF-1 components for the purpose of peptide mapping and protein microsequencing analysis. Since additional steps of purification resulted in markedly lower yield, we did not purify HIF-1 further to homogeneity. Aliquots from flowthrough of the M18 column (Fig. 4A, Load) as well as the 0.25 M KCl wash and 0.5 M KCl elute fractions of the second W18 column were analyzed by 6% SDS-PAGE and silver staining. Four polypeptides of 90-120 kDa were highly enriched in the 0.5 M KCl fraction, which had high HIF-1 DNA binding activity

compared with the 0.25 M KCl fraction, which had very little HIF-1 activity. The 0.5 M KCl fraction, however, still had many of the contaminant proteins found in the 0.25 M KCl fraction.

In an initial pilot purification of HIF-1 from hypoxia-induced Hep3B cells, a different purification protocol was used. Gel filtration over a Sephacryl S-300 column was also found to be effective in separating HIF-1 from constitutive DNA binding activity. In addition, a calf thymus DNA column was used to remove nonspecific DNA-binding proteins prior to two rounds of W18 DNA affinity chromatography. HIF-1 activity was detected in 0.5 M KCl fractions from both DNA affinity columns. An aliquot from the 0.5 M KCl elute fraction of the first W18 column (Fig. 4B, Load) as well as the 0.25 M KCl wash and 0.5 M KCl elute fractions of the second W18 column were analyzed by 7% SDS-PAGE and silver staining. Four polypeptides of similar molecular mass to those that co-purified with HIF-1 DNA binding activity in  $\text{CoCl}_2$ -treated HeLa cells were present in the affinity-purified preparation from hypoxic Hep3B cells (Fig. 4B, lane 3, arrows), indicating that HIF-1 from the two different cell types is composed of the same polypeptide subunits. Affinity-purified HIF-1 from both  $\text{CoCl}_2$ -treated HeLa cells and hypoxic Hep3B cells bound specifically to the W18 probe in gel shift assays.

**Example 3.      Analysis of HIF-1 Subunits.**

The following experiments were conducted to identify polypeptides that are part of the HIF-1 DNA binding complex.

Preparative gel shift assays were performed with 30  $\mu\text{l}$  of affinity-purified HIF-1 and probe W18. Gel slices containing HIF-1 and surrounding areas were isolated after autoradiography with wet gel. Gel slices were placed on the stacking gel of a 6% SDS-polyacrylamide gel and incubated with Laemmli buffer *in situ* for 15 min, and electrophoresis was performed in parallel with 30  $\mu\text{l}$  of affinity-purified HIF-1 and molecular weight markers. For two-dimensional denaturing gel electrophoresis, two aliquots of affinity-purified HIF-1 were resolved on a 6% SDS-polyacrylamide gel with 5% cross-linking (acrylamide/bisacrylamide ratio of 19:1). One lane was stained with silver nitrate. The gel slices corresponding to regions of interest were isolated from the unstained lane. The isolated gel slices were placed directly on the stacking gel of the second dimension 6% SDS-polyacrylamide gel with 3.2% cross-linking, and electrophoresis was performed in parallel with 30  $\mu\text{l}$  of affinity purified HIF-1.

Peptide Mapping of HIF-1 Subunits. 2 ml of the affinity-purified HIF-1 were dialyzed against 10 mM ammonium bicarbonate, 0.05% SDS and lyophilized. After resuspension in a solubilizing solution (100 mM sucrose, 3% SDS, 21.25 mM Tris-HCl (pH 6.9), 1 mM EDTA, 5%  $\beta$ -mercaptoethanol, 0.005% bromphenol blue), the protein samples were heated to 37°C for 15 min and resolved on a 6% polyacrylamide gel containing 0.2% SDS. Polypeptides were transferred electrophoretically at 4°C to a polyvinylidene difluoride membrane (Bio-Rad) in 0.5 x Towbin buffer (Towbin et al. 1979) Proc. Natl. Acad. Sci. USA 76:4350-5354) (96 mM glycine, 12.5 mM Tris-HCl (pH 8.3)) with 10% acetic acid, destained with 5% acetic acid and rinsed with Milli-Q water. Membrane slices containing the HIF-1 polypeptides of 120, 94/93, and 91 kDa were excised and subjected to peptide mapping (Best et al. (1994) in Techniques in Protein Chemistry V (Crabb, J.W., ed.), pp. 205-213, Academic Press, San Diego, CA). *In situ* tryptic digestion and reverse phase HPLC were performed by the Wistar Protein Microchemistry Laboratory.

UV Cross-Linking Analysis. UV cross-linking was carried out as described (Wang & Semenza (1993) Proc. Natl. Acad. Sci. USA 90:4304-4308) except that 30  $\mu$ l of affinity-purified HIF-1 were used in the binding reaction. Affinity-purified HIF-1 was incubated with W18 probe in the absence or presence of unlabeled W18 or M18 oligonucleotide. After incubation for 15 min at 4°C, the reaction mixtures were irradiated with UV light (312 nm; Fisher Scientific) for 30 min and resolved by 6% SDS-PAGE with pre-stained molecular weight markers and visualized by autoradiography.

Glycerol Gradient Sedimentation. Linear gradients of 12 ml, 10-30% glycerol in a buffer containing 100 mM KCl, 25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 5 mM DTT, and 0.4 mM phenylmethylsulfonyl fluoride, were prepared for centrifugation in a Beckman SW40 rotor for 48 h at 4°C. Nuclear extract prepared from hypoxic Hep3B cells (100  $\mu$ l, 5 mg/ml) was mixed with an equal volume of glycerol gradient buffer containing 10% glycerol and layered on the top of the gradient. A marker gradient was sedimented in parallel and contained 50  $\mu$ g each of thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (67 kDa) (Pharmacia). Markers were adjusted to the same volume and glycerol concentration as the sample. Fractions (0.5 ml)

were collected from the top of the tubes, and DNA binding activity was measured by the gel shift assay. Markers were assayed by SDS-PAGE and silver staining.

**Results.** In order to identify polypeptides that are part of the HIF-1 DNA binding complex, preparative gel shift assays were performed with affinity-purified HIF-1 and W18 probe. Gel slices containing the HIF-1-DNA complex were isolated, inserted directly into the wells of an SDS-polyacrylamide gel, and analyzed by electrophoresis in parallel with an aliquot of affinity-purified HIF-1 (Fig. 5A). Four polypeptides present in the HIF-1 complex migrated with an apparent molecular weight of 120, 94, 93, and 91 kDa, respectively (Fig. 5A, HIF-1). None of these peptides were detected in gel slices isolated from other regions of the same lane. These four polypeptides migrated at the same positions as the polypeptides that co-purified with HIF-1 DNA binding activity by DNA affinity chromatography (Fig. 5A, lane A). The 120 kDa polypeptide and the 91-94 kDa polypeptides appear to be present in an equimolar ratio, suggesting that the 120 kDa polypeptide forms complexes with any one of the 91-, 93-, and 94 kDa polypeptides.

On a 6% SDS-polyacrylamide gel with 3.2% cross-linking, the 120 kDa HIF-1 polypeptide migrated very close to a contaminant polypeptide of slightly greater apparent molecular weight (Fig. 5A, lane A), making isolation of the 120 kDa polypeptide difficult. This problem was resolved by separating the HIF-1 polypeptides on a 6% SDS-polyacrylamide gel with 5% cross-linking. The 120 kDa polypeptide migrated much faster on the more highly cross-linked gel relative to the migration of the 116 kDa molecular mass marker, whereas migration of the contaminant band (\*1) was unchanged (Fig. 5B, lane A). Under these conditions, however, the 91 kDa polypeptide ran very close to another contaminant band (\*2) below it. Two polyacrylamide gel systems with different degrees of crosslinking were therefore required for the isolation of the 91-94 kDa and the 120 kDa HIF-1 polypeptides, respectively.

To confirm that the HIF-1 polypeptides identified by the two gel systems were identical, two dimensional denaturing gel electrophoresis was performed. Affinity-purified HIF-1 was first resolved on a 6% SDS-polyacrylamide gel with 5% crosslinking (as in Fig. 5B, lane A). Regions of the gel containing the 120 kDa, 94/93/91-kDa HIF-1 polypeptides, as well as the two contaminant bands, were isolated and analyzed by electrophoresis on a 6% SDS-polyacrylamide gel with

3.2% crosslinking in parallel with an aliquot of the affinity-purified HIF-1. As shown in Fig. 5C, the isolated HIF-1 and contaminant polypeptides co-migrate with the corresponding bands in the control sample, indicating that the differences in their migration were due to different degrees of cross-linking of the

5 SDS-polyacrylamide gels.

To determine whether the four polypeptides from the HIF-1 complex represent distinct protein species, tryptic peptide mapping was performed. The 91 kDa band was isolated individually while the 93 and 94 kDa bands were excised together after electrophoretic separation and transfer to a polyvinylidene difluoride  
10 membrane. Proteins were digested with trypsin *in situ*, and the tryptic peptides were separated by reverse-phase HPLC (Fig. 6). The elution profiles of tryptic peptides derived from 91 kDa protein and 93/94 kDa proteins were nearly superimposable (Fig. 6), suggesting that they were derived from similar polypeptides. Another aliquot of HIF-1 was resolved on a 6% polyacrylamide gel  
15 of 5% crosslinking for isolation of the 120 kDa HIF-1 polypeptide. The tryptic peptide elution profile derived from the 120 kDa polypeptide was distinct from those of the 91-94 kDa polypeptides. These results suggest that HIF-1 is composed of two different subunits, 120 kDa HIF-1 $\alpha$  and 91/93/94 kDa HIF-1 $\beta$ .

To identify the DNA-binding subunit(s), affinity-purified HIF-1 was incubated  
20 with W18 probe. After UV irradiation to cross-link the DNA-binding proteins to nucleotide residues at the binding site, the reaction mixtures were boiled in Laemmli buffer and resolved by SDS-PAGE, and cross-linked proteins were visualized by autoradiography. Two DNA-binding proteins were detected (Fig. 7, lane 1). Their molecular masses were estimated to be approximately 120 and 92  
25 kDa (after the 16 kDa molecular mass contributed by probe DNA was subtracted), similar to those of HIF-1 $\alpha$  and HIF-1 $\beta$ . The binding of both proteins to the probe was sequence-specific since it could be competed by unlabeled wild-type W18 (Fig. 7, lane 2) but not mutant M18 (Fig. 7, lane 3) oligonucleotide. These results suggest that both HIF-1 $\alpha$  and HIF-1 $\beta$  contact DNA directly. HIF-1 $\alpha$  was  
30 cross-linked to DNA much more strongly than HIF-1 $\beta$  (fig. 7, lanes 1 and 3). These data provided further evidence that the four polypeptides purified by DNA affinity chromatography are bona fide components of HIF-1 DNA binding activity.

To estimate the native size of HIF-1, glycerol gradient sedimentation analysis was performed with crude nuclear extract prepared from hypoxic Hep3B cells.

HIF-1 and the constitutive DNA binding activity were monitored by gel shift assays. In hypoxic Hep3B nuclear extracts, HIF-1-DNA complexes are present in two forms, whereas in  $\text{CoCl}_2$ -treated HeLa extracts, the faster migrating form predominates. The results, shown in Fig. 8, demonstrate that the two bands of the HIF-1 doublet are separable by sedimentation. The faster migrating form was estimated to have a molecular mass of approximately 200-220 kDa. Longer exposure of the autoradiograph revealed that the slower migrating band co-migrated with ferritin, which has a molecular mass of 440 kDa. Assuming a globular conformation for both protein complexes, these results are consistent with the hypothesis that the faster migrating form represents a heterodimeric complex, consisting of a 120 kDa HIF-1 $\alpha$  subunit and a 91-94 kDa HIF-1 $\beta$  subunit, whereas the slower migrating form may represent a heterotetramer. The exact nature and stoichiometry of these HIF-1 complexes, however, remains to be determined. The constitutive DNA binding activity has a molecular mass less than the 67 kDa BSA protein. Since UV cross-linking analysis indicated that the constitutive factor has a DNA-binding subunit of approximately 40-50 kDa, it is most likely that the constitutive factor binds DNA as a monomer. Consistent with the results of glycerol gradient sedimentation analysis, HIF-1 eluted from a Sephacryl S-300 gel filtration column before the constitutive binding activity, and the slower migrating HIF-1 gel shift activity eluted before the faster migrating form. These results suggest that HIF-1 exists predominantly as a heterodimer in solution and to a lesser extent as a higher order complex, and that these complexes contain at least one HIF-1 $\alpha$  and one HIF-1 $\beta$  subunit.

**Example 4. Isolation and Characterization of HIF-1 $\alpha$  cDNA Sequences.**

Protein microsequence analysis. Purified HIF-1 subunits were fractionated by SDS-polyacrylamide gel electrophoresis, and the 120 and 94 kDa polypeptides were transferred to polyvinylidene difluoride membranes, individually digested with trypsin *in situ* and peptides were fractionated by reverse-phase high-pressure liquid chromatography (Wang & Semenza (1995) J. Biol. Chem. 270:1230-1237, herein specifically incorporated by reference). Protein microsequence analysis was performed at the Wistar Protein Microchemistry Laboratory, Philadelphia (Best et al. (1994) *supra*).

cDNA library construction and screening. Poly (A)+ RNA was isolated from Hep3B cells cultured for 16 h at 37°C in a chamber flushed with 1%  $\text{O}_2$ /5%

CO<sub>2</sub>/balance N<sub>2</sub>. cDNA was synthesized using oligo(dT) and random hexamer primers and bacteriophage libraries were constructed in  $\lambda$ gt11 and Uni-ZAP XR (Stratagene, La Jolla CA). cDNA libraries were screened with <sup>32</sup>P-labelled cDNA fragments by plaque hybridization as described (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, NY, herein specifically incorporated by reference).

PCR. Degenerate oligonucleotides primers were designed using codon preference rules (Lathe (1985) J. Mol. Biol. 183:1-12).  $\alpha$ F1 (5'-ATCGGATCCATCACIGA(A/G)CT(C/G)-ATGGGITATA-3') (SEQ ID NO:7) was based upon the amino terminus of HIF-1 $\alpha$  peptide 87-1 and used as a forward primer. Two nested reverse primers,  $\alpha$ R1 (5'-ATTAAGCmTGGT-(G/C)AGGTGGTCI(G/C)(A/T)GTC-3') (SEQ ID NO:8) and  $\alpha$ R2 (5'-ATTAAGCTTGCATGGTAGTA(T/C)TCATAGAT-3') (SEQ ID NO:9), were based upon the carboxy terminus of peptide 91-1. PCR was performed by: denaturation of 108 phage or 10 ng of phage DNA at 95°C for 10 min; addition of AmpliTaq (Perkin-Elmer) at 80°C; and amplification for 3 cycles at 95°C, 37°C, and 72°C (30 sec each) followed by 35 cycles at 95°C, 50°C, and 72°C (30 sec each). Nested PCR with  $\alpha$ F1/ $\alpha$ R1 and then  $\alpha$ F1/ $\alpha$ R2 generated an 86-bp fragment which was cloned into pGEM4 (Promega). For HIF-1 $\beta$  (ARNT), PCR was performed as described above using primers 5'-ATAAAGCTTGT(C/G)TA(C/T)GT-(C/G)TCIGA(C/T)TCIG-3'(SEQ ID NO:10) and 5'ATCGAATTC(C/T)TCI-GACTGIGGCTGGTT-3'(SEQ ID NO:11) which resulted in the predicted 69-bp product. For analysis of the 5' end of HIF-1 $\beta$  (ARNT), Hep3B poly(A)+ RNA was reverse-transcribed using reagents from a 5'-RACE kit (Clontech). The cDNA was used as template to amplify nt 54-425 of ARNT cDNA (Hoffman et al. (1991) supra), with 5'-TACGGATCCGCCATGGCGGCGACT-ACTGA-3' (SEQ ID NO:12) (forward primer) and nested reverse primers 5'-AGCCAGGGCACTACAGGTGGGTACC-3' (SEQ ID NO:13) and 5'GTTCCCCGCAAGGACTTCATGTGAG-3' (SEQ ID NO:14) for 35 cycles at 95°C, 60°C, and 72°C (30 sec each). PCR products were cloned into pGEM4 for nucleotide sequence analysis.

Results. The purified 120 kDa HIF-1 $\alpha$  polypeptide was digested with trypsin, peptides were fractionated by reverse-phase high-pressure liquid chromatography and fractions 87 and 92 were subjected to microsequencing. Each fraction

contained two tryptic peptides, for which virtually complete amino acid sequences were obtained: ITELMGYEPEELLGR (SEQ ID NO:15) (87-1), XIILIPSDLAXR (SEQ ID NO:16) (87-2), SIYEYYHALDSDHLTK (SEQ ID NO:17) (91-1), and SFFLR (SEQ ID NO:18) (91-2). When 87-1 and 91-1 were entered as contiguous sequences, database searches identified similarities to the *Drosophila* proteins period (PER) and single-minded (SIM), and the mammalian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) proteins, which all contain sequences of 200-350 amino acids that constitute the PAS (PER-ARNT-AHR-SIM) domain (Hoffman et al. (1991) Science 252:954-958; Citri et al. (1987) Nature 326:42-47; Burbach et al. (1992) Proc. Natl. Acad. Sci. USA 89:8185-8189; Crews et al. (1988) Cell 52:143-151; Nambu et al. (1991) Cell 67:1157-1167). Degenerate oligonucleotides were synthesized based upon the 87-1 and 91-1 sequences and used for PCR with cDNA prepared from hypoxic Hep3B cells. Nucleotide sequence analysis revealed that the cloned PCR product encoded the predicted amino acids, demonstrating that 87-1 and 91-1 were contiguous peptides.

**Example 5. Nucleotide sequence and database analysis.** Complete unambiguous double stranded nucleotide sequences were obtained by incorporation of fluorescence-labeled dideoxy nucleotides into thermal-cycle sequencing reactions using T3, T7, and custom-synthesized primers. Reactions were performed using Applied Biosystems 394 DNA Synthesizers and 373a Automated DNA Sequencers in the Genetics Core Resources Facility of The Johns Hopkins University. Protein and nucleic acid database searches were performed at the National Center for Biotechnology Information using the programs BLASTP and TBLASTN (Altschul et al. (1990) J. Mol. Biol. 215:403-410). The HIF-1 $\alpha$  cDNA nucleotide sequence and deduced amino acid sequence have been submitted to GenBank. The accession number is U22431.

**Results.** Database analysis also identified an expressed-sequence tag (EST) whose derived amino acid sequence showed similarity to bHLH-PAS proteins. We obtained the 3.6-kb cDNA from which the EST was derived, hbc025 (Takeda et al. (1993) Hum. Mol. Genet. 2:1793-1798). Complete nucleotide sequence analysis revealed that it encoded all four tryptic peptides. Another EST was identified which shared identity with hbc025 and was encoded by a 2.0-kb cDNA,



hbc120 (Takeda et al. (1993) supra). Sequence analysis of hbc120 revealed that it was co-linear with the 3' end of hbc025 (Fig. 9), differing only in the length of the poly (A) tail. The 5' end of hbc025 was used to screen a Hep3B cDNA library, resulting in the isolation of an overlapping 3.4-kb cDNA, 3.2-3, which extended to an initiator codon. The composite cDNA of 3720 bp encoded a 2478-bp open reading frame that included a translation initiation codon, a 28-bp 5'-untranslated region (5'-UTR) that contained an in-frame termination codon, and a 1211-bp 3'-UTR that ended with a canonical polyadenylation signal followed after 12 bp by 43 adenine residues. Compared to the consensus translation-initiation sequence GCC(A/G)CCATGG (SEQ ID NO:19) (Kozak (1987) Nucleic Acids Res. 15:8125-8132), the HIF-1 $\alpha$  cDNA sequence is TTCACCATGG (SEQ ID NO:20). The HIF-1 $\alpha$  cDNA open reading frame predicted a novel 826 amino acid polypeptide (Fig. 10) with a molecular mass of 93 kDa that contained a bHLH-PAS domain at its amino terminus.

Analysis of two tryptic peptides isolated from the 94 kDa HIF-1 $\beta$  polypeptide (Wang & Semenza (1995) supra) yielded partial amino acid sequences, VVYVSDSVTPVLNQPQSE (SEQ ID NO:21) and TSQFGVGSFQTPSSFSSMXLPGAPTASPGAAAY (SEQ ID NO:22). Using degenerate oligonucleotides based upon the second peptide sequence, a PCR product of the predicted size was amplified from Hep3B cDNA. Database searches identified both peptides within the sequence of ARNT, a bHLH-PAS protein previously shown to heterodimerize with AHR to form the functional dioxin receptor (Reyes et al. (1992) Science 256:1193-1195). Two isoforms of ARNT have been identified which differ by the presence or absence of a 15 amino acid sequence encoded by a 45-bp alternative exon (Hoffman et al. (1991) supra). Analysis of Hep3B RNA by reverse transcriptase-PCR revealed the presence of both sequences, as well as additional isoforms. These primary sequence differences may account for the purification of three (91,93, and 94 kDa) HIF-1 $\beta$  polypeptides (Wang & Semenza (1995) supra). The apparent molecular mass of both HIF-1 $\alpha$  and HIF-1 $\beta$  on denaturing gels was greater than the mass predicted from the cDNA sequence. For HIF-1 $\alpha$  the apparent mass was 120 kDa compared to a calculated mass of 93 kDa; for the HIF-1 $\beta$  subunits, the apparent masses were 91-94 kDa compared to calculated masses of 85 and 87 kDa for the 774 and 789 amino acid isoforms of ARNT, respectively. The HIF-1 $\alpha$  and ARNT

sequences contain multiple consensus sites for protein phosphorylation and HIF-1 has been shown to require phosphorylation for DNA binding (Wang & Semenza (1993b) supra).

5 HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) belong to different classes of bHLH domains, which consist of contiguous DNA binding (b) and dimerization (HLH) motifs. The bHLH domain of HIF-1 $\alpha$  is most similar to the other bHLHPAS proteins, SIM and AHR (Fig. 11). HIF-1 $\beta$  (ARNT) has greatest similarity to the bHLH domains found in a series of mammalian (MI, USF, L-MYC) and yeast (CP-1) proteins that bind to 5'-CACGTG-3' (SEQ ID NO:23) (Dang et al. (1992) Proc. Natl. Acad. Sci. USA 89:599-603), a sequence which resembles the HIF-1 [5'-(G/Y)ACGTGC(G/T)-3' (SEQ ID NO:24) (Semenza et al. (1994) supra)] and dioxin receptor [5'-(TIG)NGCGTG(A/C)-(G/C)A-3' (SEQ ID NO:25) (Lusska et al. (1993) J. Biol Chem. 268:6575-6580)] binding sites. These transcription factors share bHLH domains of related sequence which occur in different dimerization contexts: MI, 10 L-MYC, and USF are bHLH-leucine zipper proteins, ARNT is a bHLH-PAS protein, and CP-1 contains only a bHLH domain.

Analysis of PAS domains, which have been implicated in both ligand binding and protein dimerization (Huang et al. (1993) Nature 364:259-262; Dolwick et al. (1993) Proc. Natl. Acad. Sci. USA 90:8566-8570; Reisz-Porszasz et al. (1994) 20 Mol. Cell. Biol. 14:6075-6086), revealed that HIF-1 $\alpha$  is most similar to SIM. Our alignment established consensus sequences that include a previously unreported motif, HXXD, present in the A and B repeats of all PAS proteins (Fig. 12). We also found that KinA of *Bacillus subtilis* (Perego et al. (1989) J. Bacteriol. 171:6187-6196) contains a PAS domain at its amino terminus and is thus the first 25 procaryotic member of this protein family, indicating a remarkable degree of evolutionary conservation. KinA, like PER, possesses a PAS but not a bHLH domain and is thus unlikely to bind DNA. *B. subtilis* undergoes sporulation in response to adverse environmental conditions and KinA functions as a sensor that transmits signals via a carboxy-terminal kinase domain (Burbulys et al. (1991) 30 Cell 64, 545-552).

#### Example 6. RNA Blot Hybridization.

The expression of HIF-1 RNAs in response to inducers of HIF-1 DNA-binding activity was analyzed as follows.

Total RNA (15 ug) was fractionated by 2.2 M formaldehyde/ 1.4% agarose gel electrophoresis, transferred to nitrocellulose membranes and hybridized at 68°C in Quik-Hyb (Stratagene) to <sup>32</sup>P-labelled HIF-1α or ARNT cDNA. Gels were stained with ethidium bromide and RNA was visualized by ultraviolet illumination before and after transfer to insure equal loading and transfer, respectively, in each lane. Based upon the migration of RNA size markers (BRL-GIBCO) on the same gels, the size of HIF-1α RNA was estimated to be 3.7 ± 0.1 kb. Two ARNT RNA species were identified as previously reported (Hoffman et al. (1991) *supra*).

**Results.** When Hep3B cells were exposed to 1% O<sub>2</sub>, HIF-1α and HIF-1β (ARNT) RNA levels peaked at 1-2 h, declined to near basal levels at 8 h, and showed a secondary increase at 16 h of continuous hypoxia (Fig. 13A). In response to 75 μM CoCl<sub>2</sub>, HIF-1 RNAs peaked at 4 h, declined at 8 h, and increased again at 16 h (Fig. 13B). In cells treated with 130 μM desferrioxamine, a single peak at 1-2 h was seen (Fig. 13C). When cells were incubated at 1% O<sub>2</sub> for 4 h and then returned to 20% O<sub>2</sub>, both HIF-1α and HIF-1β RNA decreased to below basal levels within 5 min, the earliest time point assayed (Fig. 13D). These results demonstrate that, as in the case of HIF-1 DNA-binding activity (Wang & Semenza (1993b) *supra*), HIF-1 RNA levels are tightly regulated by cellular O<sub>2</sub> tension. The marked instability of HIF-1α RNA in posthypoxic cells may involve the 3'-untranslated region (3'-UTR) which contains eight AUUUA sequences (Fig. 13E) that have been identified in RNAs with short half-lives and shown to have a destabilizing effect when introduced into heterologous RNAs (Shaw & Kamen (1986) Cell 46:659-667). Seven of the HIF-1α AUUUA sequences conform to a more stringent consensus for RNA instability elements, 5'-UUUUUA(U/A)(U/A)-3' (SEQ ID NO:26) (Lagnado et al. (1994) Mol. Cell. Biol. 14:7984-7995).

#### **Example 7.      Antibody Production.**

To analyze HIF-1 protein expression, polyclonal antisera was raised against HIF-1α and HIF-1β as follows.

Rabbits were immunized with recombinant proteins in which glutathione-S-transferase (GST) was fused to amino acids 329-531 of HIF-1α or 496-789 of ARNT. To generate antibodies against HIF-1α, a 0.6 kb EcoRI fragment from hbc025 was cloned into pGEX-3X (Pharmacia) and transformed

into *E. coli* DH5 $\alpha$  cells (GIBCO-BRL). GST/HIF-1 $\alpha$  fusion protein was isolated by exposure of bacteria ( $OD_{600} = 0.8$ ) to 0.1 mM IPTG at room temperature for 1 h; sonication in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride; centrifugation at 10,000 x g for 10 min; incubation of supernatant with glutathione-agarose (Pharmacia) in the presence of 1% NP-40 for 1 h at 4°C; and elution with 5 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl. To generate antibodies against HIF-1 $\beta$ , ARNT nt 1542-2428 were amplified from Hep3B cDNA by PCR with Taq polymerase using forward primer 5'-ATAGGATCCTCAGGTCAGCTGGCACCCAG-3' (SEQ ID NO:27) and reverse primer 5'-CCAAAGCTTCTATTCTGAAAAGGGGGG-3' (SEQ ID NO:28). The product was digested with BamHI and EcoRI, to generate a fragment corresponding to ARNT nt 1542-2387, and cloned into pGEX-2T (Pharmacia). Fusion protein isolation was as described above, except that induction was with 1 mM IPTG for 2 h and binding to glutathione-agarose was in the presence of 1% Triton X-100 rather than NP-40. Fusion proteins were excised from 10% SDS/polyacrylamide gels and used to immunize New Zealand white rabbits (HRP Inc., Denver PA) according to an institutionally-approved protocol. Antibodies raised against HIF-1 $\alpha$  were affinity-purified by binding to GST/HIF-1 $\alpha$  coupled to CNBr-activated Sepharose 4B (Pharmacia).

**Results.** Antisera was used to demonstrate that the proteins encoded by the cloned HIF-1 $\alpha$  cDNA and ARNT are components of HIF-1 DNA-binding activity (Fig. 14A). When crude nuclear extracts from hypoxic cells were incubated with probe DNA and either antiserum, the HIF-1/DNA complex seen in the absence of antisera was replaced by a more slowly migrating HIF-1/DNA/antibody complex, whereas addition of preimmune sera had no effect on the HIF-1/DNA complex.

#### **Example 8.      Immunoblot analysis.**

15 ug aliquots of nuclear protein extracts were resolved on 6% SDS/polyacrylamide gels and transferred to nitrocellulose membranes in 20 mM Tris-HCl (pH 8.0), 150 mM glycine, 20% methanol. Membranes were blocked with 5% milk/TBS-T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20], incubated with affinity-purified HIF-1 $\alpha$  antibodies or HIF-1 $\beta$  antiserum diluted 1:400 or 1:5000, respectively, washed, incubated with horseradish peroxidase anti-immunoglobulin conjugate diluted 1:5000, washed, and developed with ECL

reagents (Amersham) and autoradiography. Incubations were for 1 h in 5% milk/TBS-T and washes were for a total of 30 min in TBS-T at room temperature.

Results. Immunoblot analysis revealed that the antisera detected polypeptides in crude nuclear extracts from hypoxic Hep3B or CoCl<sub>2</sub>-treated HeLa cells which co-migrated with polypeptides present in purified HIF-1 protein preparations (Fig. 14B). Analysis of nuclear and cytoplasmic extracts prepared from Hep3B cells exposed to 1% O<sub>2</sub> (Fig. 14C) revealed that peak levels of HIF-1 $\alpha$  and HIF-1 $\beta$  were present in nuclear extracts at 4-8 h of continuous hypoxia, similar to the induction kinetics of HIF-1 DNA-binding activity (Wang & Semenza (1993) J. Biol. Chem. 268:21513-21518). For HIF-1 $\alpha$ , the predominant protein species accumulating at later time points migrated to a higher position in the gel than protein present at earlier time points, suggesting that post-translational modification of HIF-1 $\alpha$  may occur. For HIF-1 $\beta$ , the 94- and 93 kDa species were resolved from the 91 kDa form but not from each other and no shifts in migration were seen. The post-hypoxic decay of HIF-1 proteins was also remarkably rapid (Fig. 14D), indicating that, as with the RNAs, these proteins are unstable in post-hypoxic cells. For both HIF-1 $\alpha$  and ARNT, 31% of all amino acids are proline, glutamic acid, serine, or threonine (PEST) residues, which have been implicated in protein instability (Rogers et al. (1986) Science 234:364-368). In HIF-1 $\alpha$ , two 20 amino acid sequences (499-518 and 581-600; Fig. 10) each contain 15 PEST residues. For HIF-1 $\beta$  (ARNT), redistribution between nuclear and cytoplasmic compartments also appeared to play a role in both the induction and decay of nuclear protein levels.

Together with our previous studies of HIF-1, the results presented here indicate that HIF-1 is a heterodimeric bHLH-PAS transcription factor consisting of a 120 kDa HIF-1 $\alpha$  subunit complexed with a 91-94 kDa HIF-1 $\beta$  (ARNT) isoform. Thus, ARNT encodes a series of common subunits utilized by both HIF-1 and the dioxin receptor, analogous to the heterodimerization of E2A gene products with various bHLH proteins (Murre et al. (1989) Cell 58:537-544). Based upon these results and the similarity of HIF-1 $\alpha$  and SIM within the bHLH-PAS domain, ARNT may also heterodimerize with SIM. In *Drosophila*, several SIM-regulated genes are characterized by enhancer elements that include 1-5 copies of the sequence 5'-(G/A)(T/A)ACGTG-3' (SEQ ID NO:29)(Wharton et al. (1994) Development 120:3563-3569). The observation that the HIF-1, dioxin receptor, and SIM

binding sites share the sequence 5'-CGTG-3' supports the hypothesis that ARNT is capable of combinatorial association with HIF-1 $\alpha$ , AHR, and SIM since this half-site is also recognized by the transcription factors with which ARNT shows greatest similarity in the bHLH domain.

5      **Example 9.      Transcriptional Regulation of Genes Encoding Glycolytic Enzymes by HIF-1.**

The involvement of HIF-1 in transcriptional regulation of genes encoding glycolytic enzymes in hypoxic cells was investigated as follows.

10      RNA analysis. Total RNA was isolated from Hep3B and HeLa cells (Chomczynski & Sacchi (1987) Anal. Biochem 162:156-159). RNA concentrations were determined by absorbance at 260 nm. Agarose gel electrophoresis, followed by ethidium bromide staining and visualization of 28 and 18 S rRNA under UV illumination, confirmed that aliquots from different preparations contained equal amounts of intact total RNA. Plasmids N-KS<sup>+</sup> and 15 H-KS<sup>+</sup>, provided by P. Maire (Institut Cochin de Genetique Moleculaire, Paris), were linearized by digestion with HindIII. Antisense RNA was synthesized by T3 RNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]ATP. 10 ug of total cellular RNA was hybridized to H or N riboprobe (3 x 10<sup>5</sup> cpm) for 3 h at 66°C and digested with RNases A and T<sub>1</sub>; protected fragments 20 were analyzed by 8 M urea, 8% polyacrylamide gel electrophoresis (Semenza et al. (1990) Mol. Cell. Biol. 10:930-938). Human phosphoglycerate kinase 1 (PGK1) cDNA from plasmid pHPGK-7e (Michelson et al. (1985) Proc. Natl. Acad. Sci. USA 82:6965-6969), obtained from American Type Culture Collection, and rat PKM cDNA from plasmid pM2PK33 (Noguchi et al. (1986) J. Biol. Chem. 25 261:13807-13812), provided by T. Noguchi (Osaka University Medical School, Osaka, Japan), were used as random-labeled probes for blot hybridizations performed in QuikHyb (Stratagene) for 1 h at 68 °C, followed by washing in 15 mM sodium chloride, 1.5 mM sodium citrate, 0.1% SDS at 50 °C. Densitometric analysis of autoradiograms was performed with an LKM Ultrosan XL laser 30 densitometer using computerized peak integration.

Electrophoretic Mobility Shift Assay (EMSA). Crude nuclear extract preparations, conditions of probe preparation, binding reactions, and gel analysis were all previously described above. Double-stranded oligonucleotides were

synthesized according to the sequences shown in Table 2 except that each oligonucleotide contained at its 5'-end the sequence 5'-GATC-3', which formed a single-stranded 5' overhang when complementary oligonucleotides were annealed. The sense strand sequence of the W18 and M18 oligonucleotides was as given above. HIF-1 was partially purified from 50 liters of CoCl<sub>2</sub>-treated HeLa cells by crude nuclear extract preparation, DEAE-Sepharose chromatography, MonoQ fast protein liquid chromatography, and DNA affinity chromatography. Incubations with crude nuclear extracts and partially purified HIF-1 contained 100 and 1 ng of denatured calf thymus DNA, respectively. Competition experiments were performed with 5 ng of unlabeled W18 or M18 oligonucleotide.

Tissue culture. Hep3B and HeLa cells were maintained in culture and treated with 1% O<sub>2</sub>, CoCl<sub>2</sub>, DFX, and cycloheximide (CHX) as described above.

Transient Expression Assay. The psvcat reporter plasmid (pCAT Promoter, Promega) contained SV40 early region promoter, bacterial chloramphenicol acetyltransferase (CAT) coding sequences, SV40 splice, and polyadenylation signals. Oligonucleotides were cloned into the BglII and BamHI sites located 5' and 3' to the transcription unit, respectively. Plasmids pNMHcat and pHcat (Concordet et al. (1991) Nucleic Acids Res. 19:4173-4180), containing human aldolase A gene sequences fused directly to CAT coding sequences, were provided by P. Maire. pSVβgal (Promega) contained bacterial lacZ coding sequences driven by the SV40 early region promoter and enhancer. Plasmids were purified by alkaline lysis and two rounds of cesium chloride density gradient centrifugation. Hep3B cells were transfected by electroporation with a Gene Pulser (Bio-Rad) at 260 V and 960 microfarads. Duplicate electroporations were pooled and split onto two 10 cm tissue culture dishes (Corning) containing 8 ml of media. Cells were allowed to recover for 24 h in a 5% CO<sub>2</sub> 95% air incubator at 37°C, the media was replaced, and one set of duplicate plates was removed to a modular incubator chamber, which was flushed with 1% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>, sealed, and placed at 37°C. Cells were harvested 72 h after transfection, and extracts were prepared for CAT and β-galactosidase activity.

Results. The human aldolase A gene (hALDA) contains four noncoding exons, N1, N2, M, and H (Maire et al. (1987) J. Mol. Biol. 197:425-438). Transcription is initiated at exons N1 and H in most tissues other than muscle. Ribonuclease protection assays of RNA isolated from cells exposed to 20 or 1%

O<sub>2</sub> for 16 h revealed 3.0- and 2.9-fold higher levels of ALDA RNA initiated from exon H in Hep3B and HeLa cells exposed to 1% O<sub>2</sub>, whereas RNA initiated from exon N1 increased only 1.7- and 1.1-fold in hypoxic Hep3B and HeLa cells, respectively, suggesting a promoter-specific response to hypoxia.

5        We next compared the expression of ALDA and phosphoglycerate kinase 1 (PGKI) RNA in Hep3B cells exposed to 1% O<sub>2</sub> for 0-16 h. Maximal induction of both ALDA and PGK1 RNA showed delayed kinetics, suggesting a requirement for protein synthesis during induction, which was confirmed by the demonstration that treatment of Hep3B cells with 100 uM CHX decreased induction of ALDA and  
10        PGK1 RNA in hypoxic cells from 6.1- and 8.2-fold to 1.6- and 1.4-fold, respectively.

Treatment of Hep3B cells for 16 h with 75 uM CoCl<sub>2</sub> or 130 uM DFX induced both ALDA and PGK1 RNA with ALDA transcripts preferentially initiated from exon H. Analysis of the same RNA samples with a probe for PKM revealed that  
15        PKM RNA was also induced by exposure of Hep3B cells to 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX. ALDA, PGK1, and PKM RNAs were also induced by treatment of HeLa cells with 1% O<sub>2</sub>, CoCl<sub>2</sub>, or DFX. PFKL RNA was not expressed at detectable levels in Hep3B or HeLa cells. These RNA analyses demonstrate that agents that induce  
20        EPO RNA and HIF-1 activity also induce ALDA, PGK1, and PKM RNA in both EPO-producing Hep3B and nonproducing HeLa cells, with a requirement for *de novo* protein synthesis, as previously demonstrated for induction of EPO RNA and HIF-1 activity (Semenza & Wang (1992) Mol. Cell. Biol. 12:5447-5454).

Nucleotide sequences of genes encoding glycolytic enzymes present in GenBank were searched for potential HIF-1 binding sites using the query sequence  
25        5'-ACGTGC-3', which contains the 4 guanine residues that contact HIF-1 in the DNA major groove (Wang & Semenza (1993b) supra). Double-stranded oligonucleotides were synthesized corresponding to 5'-flanking sequences (5'-FS) of the human PGK1 (hPGKI), human enolase 1 (hENO1), and mouse LDHA (mLDHA) genes; 5'-untranslated sequences (5'-UT) of hPGKI; and intervening  
30        sequences (IVS) of the hALDA and mPFKL genes. These oligonucleotides contained, as potential HIF-1 sites, 5'-TACGTGCT-3' (SEQ ID NO:30), 5'-GACGTGCG-3' (SEQ ID NO:31) (which was also found in hEPO 5'-FS), and 5'-CACGTGCG-3' (SEQ ID NO:32). The first sequence is identical to the previously identified HIF-1 binding site in the EPO enhancer (Semenza & Wang



(1992) supra), whereas the latter two sequences differ at the first and last nucleotides. The ability of these oligonucleotides to bind HIF-1 was tested by EMSA.

When incubated with nuclear extract prepared from Hep3B cells exposed to 1% O<sub>2</sub> for 4 h, each probe generated a DNA protein complex of similar mobility and intensity to the HIF-1 complex formed with probe W18, corresponding to nucleotides 1-18 of the hEPO 3'-FS. In contrast, none of these probes detected an HIF-1 complex in nuclear extracts from cells maintained at 20% O<sub>2</sub>, although the EMSA patterns were otherwise similar to those obtained with nuclear extracts from hypoxic cells. The DNA-protein complex migrating below the HIF-1 complex was less intense when hypoxic (compared with non-hypoxic) nuclear extracts were assayed. We have previously shown that this complex contains a constitutively expressed factor that recognizes the same DNA sequence as HIF-1 (Wang & Semenza (1993b) supra). The decreased binding of the constitutive factor may thus result from competition for binding with HIF-1 in hypoxic extracts.

EMSA was also performed with a preparation of HIF-1 from CoCl<sub>2</sub>-treated HeLa cells that was purified approximately 600-fold by DEAE-cellulose, MonoQ, and DNA affinity chromatography. Each probe bound HIF-1 in a manner that was qualitatively and quantitatively similar to the complex formed with W18. The binding of HIF-1 to these probes was sequence-specific as it could be competed by an excess of unlabeled W18 but not by mutant oligonucleotide M18, containing a 3-nucleotide substitution previously shown to eliminate HIF-1 binding and hypoxia-inducible enhancer function. Similar results were obtained when competition experiments involving W18 and M18 were performed with crude nuclear extract from hypoxic Hep3B cells. These results identify novel HIF-1 binding sites in genes encoding ALDA, ENO1, PFKL, and PGKI as well as in the hEPO 5'-FS. The 8 oligonucleotides that have been shown to specifically bind HIF-1 (Table 2) contain 3 different binding site sequences that are represented by the consensus 5'-(C/G/T)ACGTGC(G/T)-3' (SEQ ID NO:33). Given the biased method of ascertainment, it is possible that HIF-1 may recognize other sequences not represented by this consensus. In addition to the 6 HIF-1 sites from glycolytic genes, the sequence 5'-CACGTGCT-3' (SEQ ID NO:34) was also present in the hENO1 5'-FS at -786 to -793 (Gialongo et al. (1990) Eur. J. Biochem. 190:567-573) but was not tested for HIF-1 binding. Thus, a total of 7 probable HIF-1 sites

were identified in 20.7 kb of nucleotide sequence reported to GenBank for these 5 glycolytic genes. In contrast, no sequences matching the consensus HIF-1 site were identified on either DNA strand within a total of 43.5 kb, comprising the nucleotide sequences of 5 randomly chosen genes, AFP, BUP4, CREB, DHFR, and EPOR (Gibbs et al. (1987) *Biochemistry* 26:1332-1343; Kurihara et al. (1993) *Biochem. Biophys. Res. Commun.* 192:1049-1056; Meyer et al. (1993) *Endocrinology* 132:770-780; Mitchell et al. (1986) *Mol. Cell. Biol.* 6:425-440; Noguchi et al. (1991) *Blood* 78:2548-2556).

To determine whether these HIF-1 binding sites were of functional importance, transient expression essays were performed using the reporter genes described above. Reporter plasmids were cotransfected into Hep3B cells with pSV $\beta$ gal, which was included as a control for variation in transfection efficiency. Transfected cells were split among duplicate plates that were cultured in 1 or 20% O<sub>2</sub> for 48 h, CAT and  $\beta$ -galactosidase protein synthesized following transcription of reporter and control plasmids, respectively, were quantitated from cellular extracts. The basal reporter psvcat, in which transcription of CAT coding sequences was driven by the SV40 early region promoter, generated similar CAT/ $\beta$ -galactosidase values in cells cultured at 1 and 20% O<sub>2</sub>. When one (psvcatEPO1) or two (psvcatEPO2) copies of the 33-base pair hEPO 3'-FS enhancer were cloned 3' to the transcription unit, CAT/ $\beta$ -galactosidase expression was induced 4.9- and 17-fold, respectively, in cells cultured at 1% O<sub>2</sub>, consistent with previously reported results (Semenza & Wang (1992) *supra*).

HIF-1 binding site sequences from glycolytic genes were analyzed in the same assay. The mPFKL IVS-1 and hPFK1 5'-FS oligonucleotides were chosen, as they represented sequences identical to or divergent from the HIF-1 site in the hEPO 3'-FS and were located 3' or 5' to the transcription initiation site, respectively. Two copies of the 24-base pair hPGK1 5'-FS oligonucleotide were cloned 5' to the psvcat transcription unit (Fig. 15A), analogous to its location in hPGK1. Expression of pPGK2svcat was induced 5.6-fold in hypoxic cells (Fig. 15B). Three copies of the 26-base pair mPFK1 IVS-1 oligonucleotide were also cloned 5' to the psvcat transcription unit, and pPFKL3svcat mediated a 47-fold induction in hypoxic cells (Fig. 15B).

We also performed experiments with hALDA gene sequences to analyze native promoter function and to correlate sequence requirements for induction in

the transfection assay with endogenous RNA expression data. The plasmid pNMHcat (Concordet et al. (1991) supra), in which 3.5 kb from the 5'-end of hALDA (Maire et al. (1987) supra) was fused to CAT coding sequences (Fig. 15A), mediated a 5.5-fold induction in hypoxic cells (Fig. 15B). The plasmid pHcat contained 0.76 kb of hALDA sequences that are colinear with the 3'-end of pNMHcat, starting within IVS-4 and extending 5' to exon H (Fig. 15A). Deletion of exons N1, N2, and M and their flanking sequences resulted in 20-fold increased levels of CAT expression but had no significant effect on relative expression in 1% O<sub>2</sub>, as pHcat was induced 5.4-fold in hypoxic Hep3B cells (Fig. 15B). These results are consistent with the observation of (i) specific induction of hALDA transcripts initiated from exon H and (ii) the presence of a HIF-1 binding site at the 5' end of IVS-4 contained within both pNMHcat and pHcat. Thus, sequences containing HIF-1 sites from the mPFKL, hPGK1, and hALDA genes mediated hypoxia-inducible transcription in conjunction with either a native or heterologous promoter.

**Example 10.      Construction of a Dominant-Negative Variant of HIF-1 $\alpha$ .**

A HIF-1 $\alpha$  variant was constructed to investigate functional inactivation of HIF-1.

The starting construct was the HIF-1 $\alpha$  cDNA 3.2-3 cloned into the plasmid pBluescript SK-. This plasmid was digested with the restriction endonucleases NcoI and BglII to delete sequences encoding amino acids 2-28. A double-stranded oligonucleotide was inserted that contained NcoI and BglII ends to allow recirculation of the plasmid in the presence of T4 DNA ligase. The resulting construct encodes amino acids 1-3, followed by three amino acids not present in the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, alanine, and glycine), followed by amino acids 28-826 of HIF-1 $\alpha$ . This construction (pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB) allows the *in vitro* transcription (using T7 RNA polymerase) and translation of the variant form of HIF-1 $\alpha$  (HIF-1 $\alpha$  $\Delta$ NB) (SEQ ID NO:35).

To create a dominant negative form of HIF-1 $\alpha$  for expression in mammalian tissue culture cells, a Kpn I-Not I fragment encoding the variant cDNA was excised from the pBluescript vector and cloned into the mammalian expression vector pCEP4. The plasmid was digested with AflII and BamHI, treated with Klenow form of DNA polymerase to generate blunt ends, and recircularized with

T4 DNA ligase. The resulting plasmid (pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB) (SEQ ID NO:3) encodes amino acids 1-3, followed by three amino acids not present at the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, alanine, and glycine), followed by amino acids 28-391 of HIF-1 $\alpha$ , followed by three amino acids not present at the corresponding position in wild-type HIF-1 $\alpha$  (isoleucine, glutamine, and threonine). Amino acids 392-826 were deleted to increase the stability of the variant protein (HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB) expressed in cells (Fig. 16).

Results. Hep3B cells were transiently transfected with 25 ug of the reporter gene psvcatEPO2 which contains two copies of the 33-bp enhancer sequence from the human erythropoietin gene as described above. This plasmid expressed a 9-fold higher level of CAT protein when cells were cultured at 1% O<sub>2</sub> relative to 20% O<sub>2</sub>. When the cells were transfected with psvcatEPO2 and pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB, there was dose-dependent inhibition of CAT expression at 1% O<sub>2</sub>. Table 3 shows the relative induction (expression at 1% O<sub>2</sub> divided by expression at 20% O<sub>2</sub>) as a function of the amount of pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB (ug) transfected into the cells. Results are the mean of three experiments.

Expression of variant HIF-1 $\alpha$  interfered with the activation of reporter gene expression by endogenous HIF-1 produced by hypoxic cells. The residual activation seen with 40 ug variant transfection may represent cells which took up psvcatEPO2 but not pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB. The results show that the dominant-negative variant can interfere with HIF-1 function *in vivo*.

The variant protein was used in a electrophoretic mobility shift assay of binding to a double-stranded oligonucleotide probe containing the HIF-1 binding site from the EPO enhancer. pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB was used as a template for *in vitro* transcription and translation. As increasing amounts of pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB were added to reactions containing a constant amount of templates for wild-type HIF-1 $\alpha$  and HIF-1 $\beta$ , there was a dose-dependent inhibition of DNA-binding such that when pBluescript/HIF-1 $\alpha$ 3.2T7 $\Delta$ NB was present in a 16-fold excess over the wild-type template pBluescript/HIF-1 $\alpha$ 3.2T7, HIF-1 DNA-binding was eliminated.

These *in vitro* and *in vivo* experiments demonstrate that deletion of the basic domain of HIF-1 $\alpha$  results in a protein that can block HIF-1 activity by inhibiting DNA binding.

TABLE 1. BIOCHEMICAL PURIFICATION OF HIF-1

Purification Step	Volume (ml)	Protein (mg)	HIF-1 Activity	Specific Activity	Yield (%)	Purification
HeLa nuclear Extract	435	3,040	608,000	0.2 U/ $\mu$ g	100	1
DEAE-Sepharose	240	550	440,000	0.8 U/mg	72	4
W18 DNA affinity 1	25	2.5	400,000	160	66	800
M18 DNA column	40	1.4	226,000	190	44	950
W18 DNA affinity 2	9	0.06	135,000	2,250	22	11,250

TABLE 2. OLIGONUCLEOTIDE SEQUENCES FROM EPO AND GLYCOLYTIC ENZYME GENES.

SEQUENCE	LOCATION	COORDINATES
gccc TACGTGCT gtctcacacagcctgtctga	hEPO 3'-FS	+3065/+3097
ccgggtagctggcg TACGTGCT gcag	mPFKL IVS-1	+336/+361
ggggctgctgca GACGTGCG tgtg	hEPO 5'-FS	-155/-178
gtga GACGTGCG gcttcggtttg	hPGK1 5'-FS	-172/-194
ctgcc GACGTGCG ctccggag	hPGK1 5'-UT	+31/+11
gtgggagcccagcg GACGTGCG ggaa	mLDHA 5'-FS	-75/-50
ggc CADGTGCG ccgcctgcgcctgcg	hENO1 5'-FS	-585/-610
ctt CACGTGCG gggaccagggaccgt	hALDA IVS-4	+125/+150

TABLE 3. RELATIVE INDUCTION OF REPORTER GENE IN THE PRESENCE OF HIF-1 $\alpha$  VARIANT.

ug Variant	Relative Hypoxic Induction
0	9.09
5	6.06
10	4.10
20	2.81
40	2.31

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: The Johns Hopkins University School of Medicine

(ii) TITLE OF INVENTION: HYPOXIA INDUCIBLE FACTOR-1 AND METHOD OF USE

5 (iii) NUMBER OF SEQUENCES: 35

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Fish & Richardson P.C.  
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 (C) CITY: La Jolla  
 (D) STATE: CA  
 (E) COUNTRY: USA  
 (F) ZIP: 92037

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

20 (A) APPLICATION NUMBER: PCT/US96/  
 (B) FILING DATE: 06-JUN-1995  
 (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25 (A) NAME: Haile, Lisa A.  
 (B) REGISTRATION NUMBER: 38,347  
 (C) REFERENCE/DOCKET NUMBER: 07265/053WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619/678-5070  
 (B) TELEFAX: 619/678-5099

30 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 3736 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

40 GTGAAGACAT CGCGGGGACC GATTCACC ATG GAG GGC GCC GGC GGC GCG AAC 52  
 Met Glu Gly Ala Gly Gly Ala Asn  
 1 5

GAC AAG AAA AAG ATA AGT TCT GAA CGT CGA AAA GAA AAG TCT CGA GAT 100  
 Asp Lys Lys Lys Ile Ser Ser Glu Arg Arg Lys Glu Lys Ser Arg Asp  
 10 15 20

45 GCA GCC AGA TCT CGG CGA AGT AAA GAA TCT GAA GTT TTT TAT GAG CTT 148  
 Ala Ala Arg Ser Arg Arg Ser Lys Glu Ser Glu Val Phe Tyr Glu Leu  
 25 30 35 40

- 50 -

	GCT CAT CAG TTG CCA CTT CCA CAT AAT GTG AGT TCG CAT CTT GAT AAG Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His Leu Asp Lys 45 50 55	196
5	GCC TCT GTG ATG AGG CTT ACC ATC AGC TAT TTG CGT GTG AGG AAA CTT Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val Arg Lys Leu 60 65 70	244
	CTG GAT GCT GGT GAT TTG GAT ATT GAA GAT GAC ATG AAA GCA CAG ATG Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys Ala Gln Met 75 80 85	292
10	AAT TGC TTT TAT TTG AAA GCC TTG GAT GGT TTT GTT ATG GTT CTC ACA Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met Val Leu Thr 90 95 100	340
15	GAT GAT GGT GAC ATG ATT TAC ATT TCT GAT AAT GTG AAC AAA TAC ATG Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn Lys Tyr Met 105 110 115 120	388
	GGA TTA ACT CAG TTT GAA CTA ACT GGA CAC AGT GTG TTT GAT TTT ACT Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe Asp Phe Thr 125 130 135	436
20	CAT CCA TGT GAC CAT GAG GAA ATG AGA GAA ATG CTT ACA CAC AGA AAT His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr His Arg Asn 140 145 150	484
	GGC CTT GTG AAA AAG GGT AAA GAA CAA AAC ACA CAG CGA AGC TTT TTT Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg Ser Phe Phe 155 160 165	532
25	CTC AGA ATG AAG TGT ACC CTA ACT AGC CGA GGA AGA ACT ATG AAC ATA Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr Met Asn Ile 170 175 180	580
30	AAG TCT GCA ACA TGG AAG GTA TTG CAC TGC ACA GGC CAC ATT CAC GTA Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His Ile His Val 185 190 195 200	628
	TAT GAT ACC AAC AGT AAC CAA CCT CAG TGT GGG TAT AAG AAA CCA CCT Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys Lys Pro Pro 205 210 215	676
35	ATG ACC TGC TTG GTG CTG ATT TGT GAA CCC ATT CCT CAC CCA TCA AAT Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His Pro Ser Asn 220 225 230	724
	ATT GAA ATT CCT TTA GAT AGC AAG ACT TTC CTC AGT CGA CAC AGC CTG Ile Glu Ile Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg His Ser Leu 235 240 245	772
40	GAT ATG AAA TTT TCT TAT TGT GAT GAA AGA ATT ACC GAA TTG ATG GGA Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu Leu Met Gly 250 255 260	820
45	TAT GAG CCA GAA GAA CTT TTA GGC CGC TCA ATT TAT GAA TAT TAT CAT Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu Tyr Tyr His 265 270 275 280	868



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	GCT TTG GAC TCT GAT CAT CTG ACC AAA ACT CAT CAT GAT ATG TTT ACT	916
	Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp Met Phe Thr	
	285 290 295	
5	AAA GGA CAA GTC ACC ACA GGA CAG TAC AGG ATG CTT GCC AAA AGA GGT	964
	Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala Lys Arg Gly	
	300 305 310	
	GGA TAT GTC TGG GTT GAA ACT CAA GCA ACT GTC ATA TAT AAC ACC AAG	1012
	Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr Asn Thr Lys	
	315 320 325	
10	AAT TCT CAA CCA CAG TGC ATT GTA TGT GTG AAT TAC GTT GTG AGT GGT	1060
	Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val Val Ser Gly	
	330 335 340	
	ATT ATT CAG CAC GAC TTG ATT TTC TCC CTT CAA CAA ACA GAA TGT GTC	1108
	Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr Glu Cys Val	
15	345 350 355 360	
	CTT AAA CCG GTT GAA TCT TCA GAT ATG AAA ATG ACT CAG CTA TTC ACC	1156
	Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln Leu Phe Thr	
	365 370 375	
20	AAA GTT GAA TCA GAA GAT ACA AGT AGC CTC TTT GAC AAA CTT AAG AAG	1204
	Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys Leu Lys Lys	
	380 385 390	
	GAA CCT GAT GCT TTA ACT TTG CTG GCC CCA GCC GCT GGA GAC ACA ATC	1252
	Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly Asp Thr Ile	
	395 400 405	
25	ATA TCT TTA GAT TTT GGC AGC AAC GAC ACA GAA ACT GAT GAC CAG CAA	1300
	Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp Asp Gln Gln	
	410 415 420	
	CTT GAG GAA GTA CCA TTA TAT AAT GAT GTA ATG CTC CCC TCA CCC AAC	1348
	Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro Ser Pro Asn	
30	425 430 435 440	
	GAA AAA TTA CAG AAT ATA AAT TTG GCA ATG TCT CCA TTA CCC ACC GCT	1396
	Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu Pro Thr Ala	
	445 450 455	
35	GAA ACG CCA AAG CCA CTT CGA AGT AGT GCT GAC CCT GCA CTC AAT CAA	1444
	Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala Leu Asn Gln	
	460 465 470	
	GAA GTT GCA TTA AAA TTA GAA CCA AAT CCA GAG TCA CTG GAA CTT TCT	1492
	Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu Glu Leu Ser	
	475 480 485	
40	TTT ACC ATG CCC CAG ATT CAG GAT CAG ACA CCT AGT CCT TCC GAT GGA	1540
	Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro Ser Asp Gly	
	490 495 500	
45	AGC ACT AGA CAA AGT TCA CCT GAG CCT AAT AGT CCC AGT GAA TAT TGT	1588
	Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser Glu Tyr Cys	
	505 510 515 520	

- 52 -

	TTT TAT GTG GAT AGT GAT ATG GTC AAT GAA TTC AAG TTG GAA TTG GTA Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val 525 530 535	1636
5	GAA AAA CTT TTT GCT GAA GAC ACA GAA GCA AAG AAC CCA TTT TCT ACT Glu Lys Leu Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr 540 545 550	1684
	CAG GAC ACA GAT TTA GAC TTG GAG ATG TTA GCT CCC TAT ATC CCA ATG Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met 555 560 565	1732
10	GAT GAT GAC TTC CAG TTA CGT TCC TTC GAT CAG TTG TCA CCA TTA GAA Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu 570 575 580	1780
15	AGC AGT TCC GCA AGC CCT GAA AGC GCA AGT CCT CAA AGC ACA GTT ACA Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr 585 590 595 600	1828
	GTA TTC CAG CAG ACT CAA ATA CAA GAA CCT ACT GCT AAT GCC ACC ACT Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr 605 610 615	1876
20	ACC ACT GCC ACC ACT GAT GAA TTA AAA ACA GTG ACA AAA GAC CGT ATG Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met 620 625 630	1924
	GAA GAC ATT AAA ATA TTG ATT GCA TCT CCA TCT CCT ACC CAC ATA CAT Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr His Ile His 635 640 645	1972
25	AAA GAA ACT ACT AGT GCC ACA TCA TCA CCA TAT AGA GAT ACT CAA AGT Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp Thr Gln Ser 650 655 660	2020
30	CGG ACA GCC TCA CCA AAC AGA GCA GGA AAA GGA GTC ATA GAA CAG ACA Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile Glu Gln Thr 665 670 675 680	2068
	GAA AAA TCT CAT CCA AGA AGC CCT AAC GTG TTA TCT GTC GCT TTG AGT Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val Ala Leu Ser 685 690 695	2116
35	CAA AGA ACT ACA GTT CCT GAG GAA GAA CTA AAT CCA AAG ATA CTA GCT Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys Ile Leu Ala 700 705 710	2164
	TTG CAG AAT GCT CAG AGA AAG CGA AAA ATG GAA CAT GAT GGT TCA CTT Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp Gly Ser Leu 715 720 725	2212
40	TTT CAA GCA GTA GGA ATT GGA ACA TTA TTA CAG CAG CCA GAC GAT CAT Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Gln Pro Asp Asp His 730 735 740	2260
45	GCA GCT ACT ACA TCA CTT TCT TGG AAA CGT GTA AAA GGA TGC AAA TCT Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly Cys Lys Ser 745 750 755 760	2308

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	AGT GAA CAG AAT GGA ATG GAG CAA AAG ACA ATT ATT TTA ATA CCC TCT	2356
	Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu Ile Pro Ser	
	765 770 775	
5	GAT TTA GCA TGT AGA CTG CTG GGG CAA TCA ATG GAT GAA AGT GGA TTA	2404
	Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu Ser Gly Leu	
	780 785 790	
	CCA CAG CTG ACC AGT TAT GAT TGT GAA GTT AAT GCT CCT ATA CAA GGC	2452
	Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln Gly	
	795 800 805	
10	AGC AGA AAC CTA CTG CAG GGT GAA GAA TTA CTC AGA GCT TTG GAT CAA	2500
	Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala Leu Asp Gln	
	810 815 820	
	GTT AAC T GAGCTTTTTC TTAATTTTCAT TCCTTTTTTTT GGACACTGGT GGCTCACTAC	2557
15	Val Asn	
	825	
	CTAAAGCAGT CTATTTATAT TTTCTACATC TAATTTTAGA AGCCTGGCTA CAATACTGCA	2617
	CAAACCTTGGT TAGTTCAATT TTTGATCCCC TTTCTACTTA ATTTACATTA ATGCTCTTTT	2677
	TTAGTATGTT CTTTAATGCT GGATCACAGA CAGCTCATTT TCTCAGTTTT TTGGTATTTA	2737
	AACCATTGCA TTGCAGTAGC ATCATTAAAT AAAAAATGCA CCTTTTATT TATTTATTTT	2797
20	TGGCTAGGGA GTTTATCCCT TTTTCGAATT ATTTTAAAGA AGATGCCAAT ATAATTTTTG	2857
	TAAGAAGGCA GTAACCTTTC ATCATGATCA TAGGCAGTTG AAAAAATTTT ACACCTTTTT	2917
	TTTCACAAAT TTTACATAAA TAATAATGCT TTGCCAGCAG TACGTGGTAG CCACAATTGC	2977
	ACAATATATT TTCTTAAAAA ATACCAGCAG TTAATCATGG AATATATTCT GCGTTTATAA	3037
	AACTAGTTTT TAAGAAGAAA TTTTTTTTGG CCTATGAAAT TGTAAACAA CTGGAACATG	3097
25	ACATTGTAA TCATATAATA ATGATTCTTA AATGCTGTAT GGTTTATTAT TTAAATGGGT	3157
	AAAGCCATTT ACATAATATA GAAAGATATG CATATATCTA GAAGGTATGT GGCATTTATT	3217
	TGGATAAAAT TCTCAATTCA GAGAAATCAA ATCTGATGTT TCTATAGTCA CTTTGCCAGC	3277
	TCAAAAGAAA ACAATACCCT ATGTAGTTGT GGAAGTTTAT GCTAATATTG TGTAAGTATG	3337
	ATTAAACCTA AATGTTCTGC CTACCCTGTT GGTATAAAGA TATTTTGAGC AGACTGTAAA	3397
30	CAAGAAAAAA AAAAAATCAT GCATTCTTAG CAAAATTGCC TAGTATGTTA ATTTGCTCAA	3457
	AATACAATGT TTGATTTTAT GCACTTTGTC GCTATTAACA TCCTTTTTTTT CATGTAGATT	3517
	TCAATAATTG AGTAATTTTA GAAGCATTAT TTTAGGAATA TATAGTTGTC AAAAACAGTA	3577
	AATATCTTGT TTTTCTATG TACATTGTAC AAATTTTCA TTCCTTTTGC TCTTTGTGGT	3637
	TGGATCTAAC ACTAACTGTA TTGTTTTGTT ACATCAAATA AACATCTTCT GTGGAAAAAA	3697
35	AAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	3736

- 54 -

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 826 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

5

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu  
 1 5 10 15

Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys  
 20 25 30

Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His  
 35 40 45

Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile  
 50 55 60

Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile  
 65 70 75 80

Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu  
 85 90 95

Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile  
 100 105 110

Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr  
 115 120 125

Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met  
 130 135 140

Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu  
 145 150 155 160

Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr  
 165 170 175

Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu  
 180 185 190

His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro  
 195 200 205

Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys  
 210 215 220

Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys  
 225 230 235 240

Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp  
 245 250 255

Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly  
 260 265 270

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	Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr	275	280	285
	Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln	290	295	300
5	Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln	305	310	315
	Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val	325	330	335
10	Cys Val Asn Tyr Val Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe	340	345	350
	Ser Leu Gln Gln Thr Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp	355	360	365
	Met Lys Met Thr Gln Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser	370	375	380
15	Ser Leu Phe Asp Lys Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu	385	390	395
	Ala Pro Ala Ala Gly Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn	405	410	415
20	Asp Thr Glu Thr Asp Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn	420	425	430
	Asp Val Met Leu Pro Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu	435	440	445
	Ala Met Ser Pro Leu Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser	450	455	460
25	Ser Ala Asp Pro Ala Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro	465	470	475
	Asn Pro Glu Ser Leu Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp	485	490	495
30	Gln Thr Pro Ser Pro Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu	500	505	510
	Pro Asn Ser Pro Ser Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val	515	520	525
	Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr	530	535	540
35	Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu	545	550	555
	Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser	565	570	575
40	Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser	580	585	590

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Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln  
595 600 605

Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu  
610 615 620

5 Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala  
625 630 635 640

Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser  
645 650 655

10 Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala  
660 665 670

Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro  
675 680 685

Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu  
690 695 700

15 Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg  
705 710 715 720

Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr  
725 730 735

20 Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp  
740 745 750

Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln  
755 760 765

Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly  
770 775 780

25 Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys  
785 790 795 800

Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu  
805 810 815

30 Glu Leu Leu Arg Ala Leu Asp Gln Val Asn  
820 825

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 373 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40 Met Glu Gly Ile Ala Gly Ser Arg Arg Ser Lys Glu Ser Glu Val Phe  
1 5 10 15

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Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His  
 20 25 30  
 Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val  
 35 40 45  
 5 Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys  
 50 55 60  
 Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met  
 65 70 75 80  
 10 Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn  
 85 90 95  
 Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe  
 100 105 110  
 Asp Phe Thr His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr  
 115 120 125  
 15 His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg  
 130 135 140  
 Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr  
 145 150 155 160  
 20 Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His  
 165 170 175  
 Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys  
 180 185 190  
 Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His  
 195 200 205  
 25 Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg  
 210 215 220  
 His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu  
 225 230 235 240  
 30 Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu  
 245 250 255  
 Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp  
 260 265 270  
 Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala  
 275 280 285  
 35 Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr  
 290 295 300  
 Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val  
 305 310 315 320  
 40 Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr  
 325 330 335

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Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln  
 340 345 350

Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys  
 355 360 365

5 Leu Lys Ile Gln Thr  
 370

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 805 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 Met Glu Gly Ile Ala Gly Ser Arg Arg Ser Lys Glu Ser Glu Val Phe  
 1 5 10 15

Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His  
 20 25 30

20 Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val  
 35 40 45

Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys  
 50 55 60

Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met  
 65 70 75 80

25 Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn  
 85 90 95

Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe  
 100 105 110

30 Asp Phe Thr His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr  
 115 120 125

His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg  
 130 135 140

Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr  
 145 150 155 160

35 Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His  
 165 170 175

Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys  
 180 185 190

40 Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His  
 195 200 205



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	Pro Ser Asn Ile Glu Ile	Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg
	210	215 220
	His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu	
	225	230 235 240
5	Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu	
		245 250 255
	Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp	
		260 265 270
10	Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala	
		275 280 285
	Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr	
		290 295 300
	Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val	
		305 310 315 320
15	Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr	
		325 330 335
	Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln	
		340 345 350
20	Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys	
		355 360 365
	Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly	
		370 375 380
	Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp	
		385 390 395 400
25	Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro	
		405 410 415
	Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu	
		420 425 430
30	Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala	
		435 440 445
	Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu	
		450 455 460
	Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro	
		465 470 475 480
35	Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser	
		485 490 495
	Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu	
		500 505 510
40	Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro	
		515 520 525

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Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr  
 530 535 540  
 Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser  
 545 550 555 560  
 5 Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser  
 565 570 575  
 Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn  
 580 585 590  
 10 Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys  
 595 600 605  
 Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr  
 610 615 620  
 His Ile His Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp  
 625 630 635 640  
 15 Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile  
 645 650 655  
 Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val  
 660 665 670  
 20 Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys  
 675 680 685  
 Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp  
 690 695 700  
 Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Gln Pro  
 705 710 715 720  
 25 Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly  
 725 730 735  
 Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu  
 740 745 750  
 30 Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu  
 755 760 765  
 Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro  
 770 775 780  
 Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala  
 785 790 795 800  
 35 Leu Asp Gln Val Asn  
 805

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCGCCCTA CGTGCTGTCT CA

22

(2) INFORMATION FOR SEQ ID NO:6:

5

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCGCCCTA AAAGCTGTCT CA -

22

(2) INFORMATION FOR SEQ ID NO:7:

15

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20

(ix) FEATURE:

(D) OTHER INFORMATION: N at positions 15 and 27 is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCGGATCCA TCACNGARCT SATGGGNTAT A

31

(2) INFORMATION FOR SEQ ID NO:8:

25

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(D) OTHER INFORMATION: N is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATTAAGCMTG GTSAGGTGGT CNSWGTC

27

35

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTAAGCTTG CATGGTAGTA YTCATAGAT

29

(2) INFORMATION FOR SEQ ID NO:10:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(D) OTHER INFORMATION: N is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAAAGCTTG TSTAYGTSTC NGAYTCGG

28

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(D) OTHER INFORMATION: N is inosine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25

ATCGAATTCY TCNGACTGNG GCTGGTT

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACGGATCCG CCATGGCGGC GACTACTGA

29

35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

- 63 -

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCCAGGGCA CTACAGGTGG GTACC

25

(2) INFORMATION FOR SEQ ID NO:14:

5

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTCCCCGCA AGGACTTCAT GTGAG

25

(2) INFORMATION FOR SEQ ID NO:15:

15

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ile	Thr	Glu	Leu	Met	Gly	Tyr	Glu	Pro	Glu	Glu	Leu	Leu	Gly	Arg
1				5				10					15	

(2) INFORMATION FOR SEQ ID NO:16:

25

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Xaa	Ile	Ile	Leu	Ile	Pro	Ser	Asp	Leu	Ala	Xaa	Arg
1				5				10			

(2) INFORMATION FOR SEQ ID NO:17:

35

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser	Ile	Tyr	Glu	Tyr	Tyr	His	Ala	Leu	Asp	Ser	Asp	His	Leu	Thr	Lys
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:18:

5

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser	Phe	Phe	Leu	Arg
1			5	

(2) INFORMATION FOR SEQ ID NO:19:

15

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCRCATGG

10

(2) INFORMATION FOR SEQ ID NO:20:

25

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCACCATGG

10

(2) INFORMATION FOR SEQ ID NO:21:

35

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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Val Val Tyr Val Ser Asp Ser Val Thr Pro Val Leu Asn Gln Pro Gln  
 1 5 10 15  
 Ser Glu

5 (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Thr Ser Gln Phe Gly Val Gly Ser Phe Gln Thr Pro Ser Ser Phe Ser  
 1 5 10 15

15

Ser Met Xaa Leu Pro Gly Ala Pro Thr Ala Ser Pro Gly Ala Ala Ala  
 20 25 30

Tyr

(2) INFORMATION FOR SEQ ID NO:23:

20

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 6 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CACGTG

6

(2) INFORMATION FOR SEQ ID NO:24:

30

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 7 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

BACGTGC

7

(2) INFORMATION FOR SEQ ID NO:25:

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- 5 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 12 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:  
    (D) OTHER INFORMATION: N is inosine.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
- 10 TNGNGCGTGM SA 12
- (2) INFORMATION FOR SEQ ID NO:26:
- 15 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 9 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
- UUAUUUAWW 9
- 20 (2) INFORMATION FOR SEQ ID NO:27:
- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 29 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
- ATAGGATCCT CAGGTCAGCT GGCACCCAG 29
- (2) INFORMATION FOR SEQ ID NO:28:
- 30 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 27 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
- CCTAAGCTTC TATTCTGAAA AGGGGGG 27
- (2) INFORMATION FOR SEQ ID NO:29:



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5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:  
RWACGTG 7

(2) INFORMATION FOR SEQ ID NO:30:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:  
TACGTGCT 8

(2) INFORMATION FOR SEQ ID NO:31:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
GACGTGCG 8

(2) INFORMATION FOR SEQ ID NO:32:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:  
CACGTGCG 8

(2) INFORMATION FOR SEQ ID NO:33:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

BACGTGCK

8

(2) INFORMATION FOR SEQ ID NO:34:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CACGTGCT

8

(2) INFORMATION FOR SEQ ID NO:35:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met	Glu	Gly	Ile	Ala	Gly	Ala	Asn	Asp	Lys	Lys	Lys	Ile	Ser	Ser	Glu
1				5					10				15		

Arg	Arg	Lys	Glu	Lys	Ser	Arg	Asp	Ala	Ala	Arg	Ser	Arg	Arg
			20					25					30

25

## Claims

1. Purified human HIF-1.
2. The human HIF-1 $\alpha$  polypeptide encoded by
  - (a) the DNA sequence set out in Fig. 10 (SEQ ID NO:1) or its  
5 complementary strand; and
  - (b) DNA sequences which hybridize under stringent conditions to the DNA sequences defined in (a).
3. An isolated nucleotide sequence encoding the human HIF-1 $\alpha$  polypeptide.
- 10 4. The isolated nucleotide sequence of claim 3 selected from the group consisting of:
  - (a) SEQ ID NO:1;
  - (b) nucleic acid sequences complementary to SEQ ID NO:1;
  - (c) fragments of (a) or (b) that are at least 15 bases in length and that will  
15 selectively hybridize to nucleotides which encode the HIF-1 $\alpha$  polypeptide of SEQ ID NO:1, under stringent conditions.
5. The nucleotide of claim 3, wherein the nucleotide is isolated from a mammalian cell.
- 20 6. The nucleotide of claim 5, wherein the mammalian cell is a human cell.
7. An expression vector including the nucleotide of claim 3.
8. The vector of claim 7, wherein the vector is a plasmid.
9. The vector of claim 7, wherein the vector is a virus.
10. A host cell stably transformed with the vector of claim 7.

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11. The host cell of claim 10, wherein the cell is prokaryotic.
12. The host cell of claim 10, wherein the cell is eukaryotic.
13. A purified antibody that binds to HIF-1 or to the HIF-1 $\alpha$  polypeptide or immunoreactive fragments thereof.
- 5 14. The antibody of claim 13, wherein the antibody is polyclonal.
15. The antibody of claim 13, wherein the antibody is monoclonal.
16. A purified and isolated nucleotide sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of HIF-1 $\alpha$  to allow possession of the biological activities of promoting the synthesis of erythropoietin (EPO), aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), pyruvate kinase M (PKM) and vascular endothelial growth factor (VEGF) in Hep3B cells.
- 10 17. A human HIF-1 $\alpha$  variant polypeptide which dimerizes with an HIF-1 $\beta$  isoform wherein at least one of the amino acids of SEQ ID NO:2 is replaced by another amino acid.
- 15 18. An isolated nucleotide sequence encoding the human variant HIF-1 $\alpha$  polypeptide having the sequence of SEQ ID NO:4.
19. A method of detecting HIF-1 $\alpha$  comprising contacting a specimen of a subject with a reagent that binds HIF-1 $\alpha$  and detecting binding of the reagent to HIF-1 $\alpha$ .
- 20 20. The method of claim 19 wherein the reagent is a nucleotide sequence complementary to SEQ ID NO:1 or a portion thereof.
21. The method of claim 18 wherein the reagent is an antibody specific for HIF-1 $\alpha$ .

22. A method for enhancing expression of a structural genetic sequence whose regulatory region contains an HIF-1 binding site, comprising administering a therapeutically effective amount of a nucleotide sequence encoding HIF-1 $\alpha$ , whereby expression of the structural genetic sequence is enhanced.

5           23. The method of claim 22, wherein the structural genetic sequence encodes EPO.

24. The method of claim 22, wherein the structural genetic sequence encodes VEGF.

10           25. The method of claim 22, wherein the structural genetic sequence encodes a glycolytic enzyme.

26. A method of treating hypoxia-related tissue damage in a subject in need thereof, comprising administering a therapeutically effective amount of a nucleotide sequence encoding HIF-1 $\alpha$ , wherein tissue damage is substantially inhibited.

27. A method of treating hypoxia-related tissue damage in a subject in need thereof, comprising introducing a nucleotide sequence of claim 3 into cells of the subject, wherein a therapeutically effective amount of HIF-1 $\alpha$  is expressed in the subject, wherein tissue damage is substantially inhibited.

5 28. A method for inhibiting expression of a structural genetic sequence whose regulatory region contains an HIF-1 binding site, comprising administering a therapeutically effective amount of an inhibitory nucleotide sequence, whereby expression of the structural genetic sequence is inhibited.

10 29. The method of claim 28 wherein the inhibitory nucleotide sequence hybridizes to an HIF-1 $\alpha$  encoding nucleotide sequence.

30. The method of claim 29, wherein the HIF-1 $\alpha$  encoding nucleotide sequence is RNA.

31. The method of claim 29, wherein the HIF-1 $\alpha$  encoding nucleotide sequence is DNA.

15 32. The method of claim 28 wherein the inhibitory nucleotide sequence encodes an HIF-1 $\alpha$  variant polypeptide.

33. A pharmaceutical composition comprising a pharmaceutically acceptable carrier admixed with a therapeutically effective amount of HIF-1.

20 34. A pharmaceutical composition comprising a nucleotide sequence encoding HIF-1 $\alpha$  in a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising an HIF-1 $\alpha$  inhibitory nucleotide sequence in a pharmaceutically acceptable carrier.

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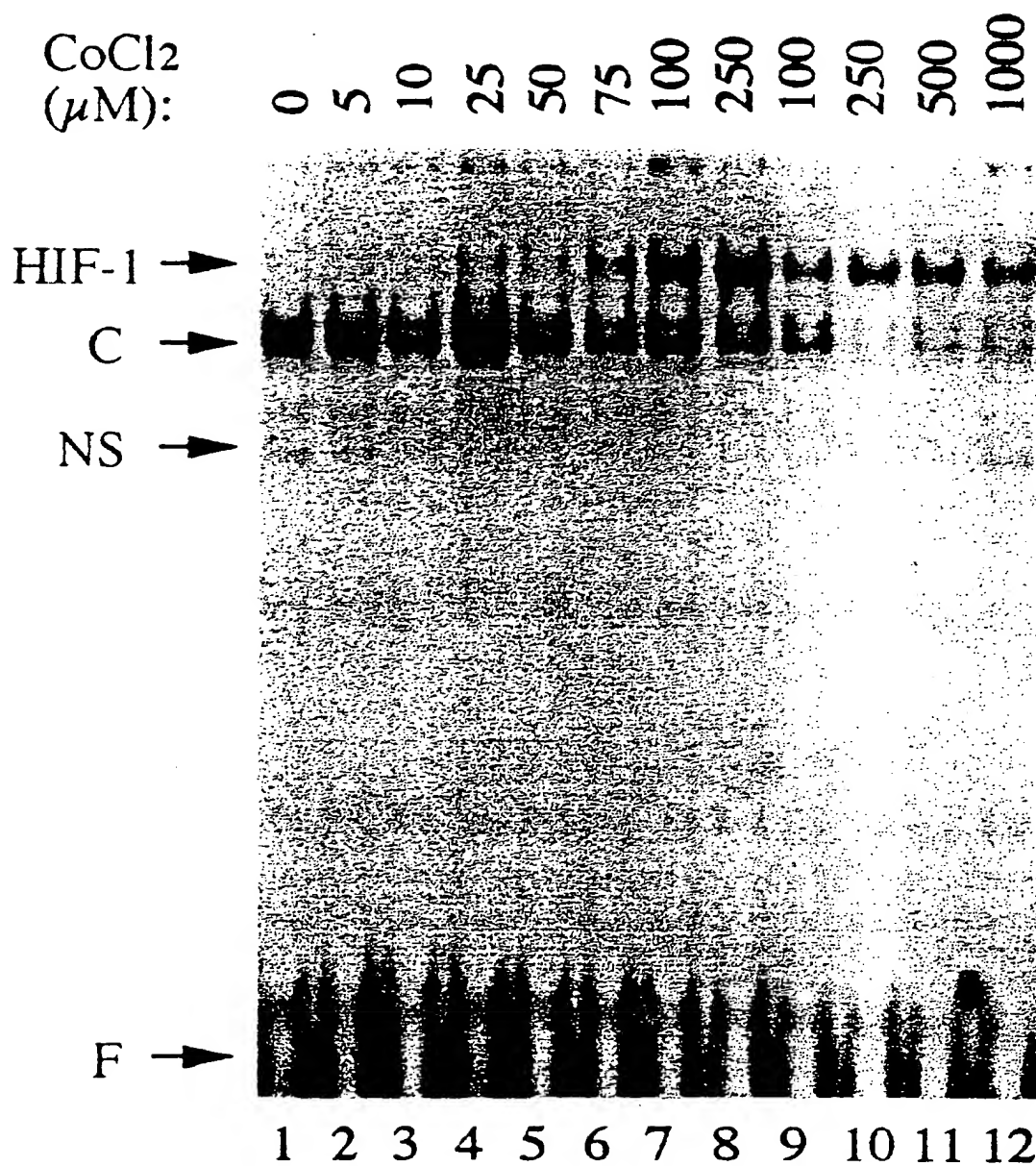


FIG. 1

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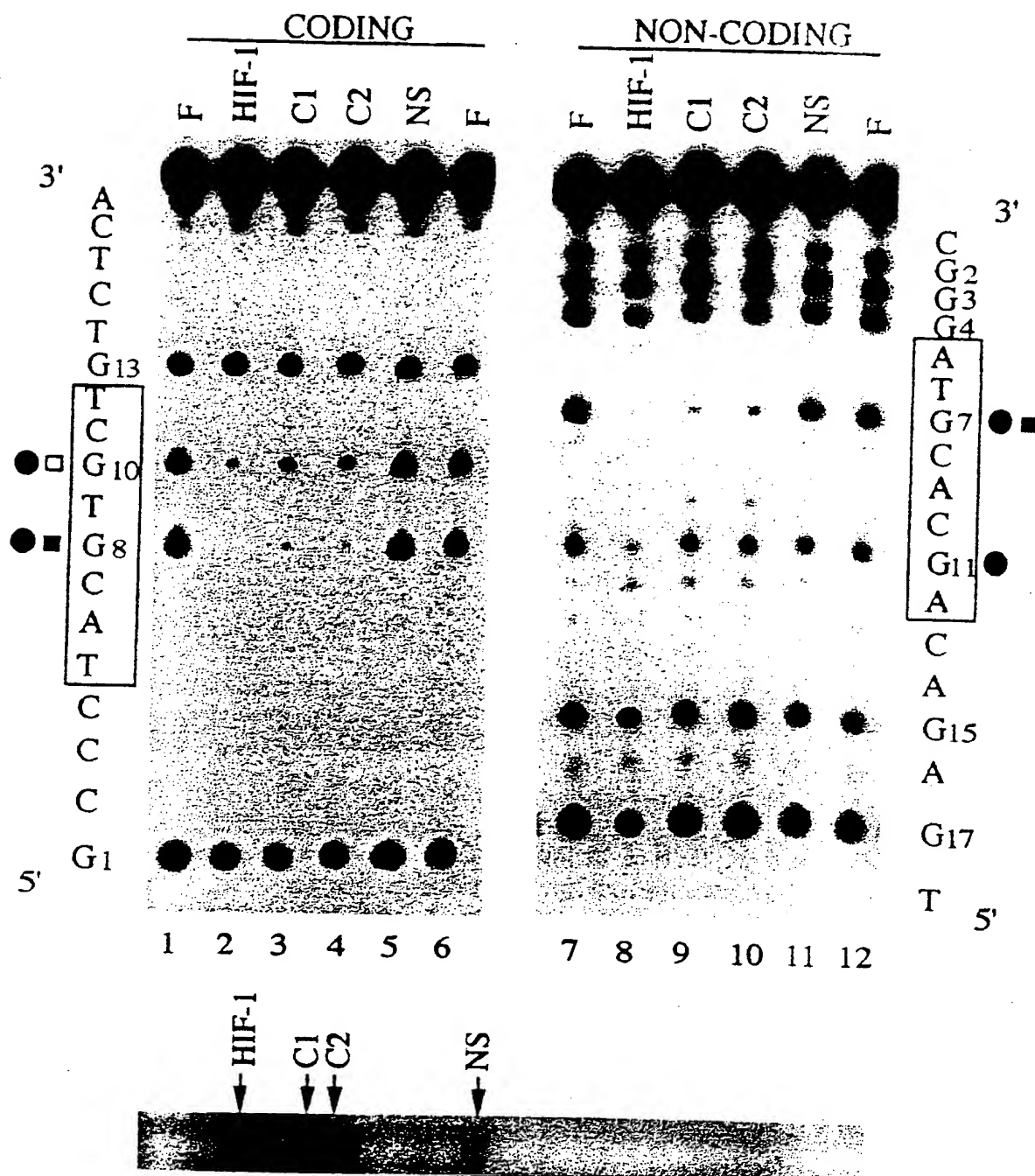


FIG. 2

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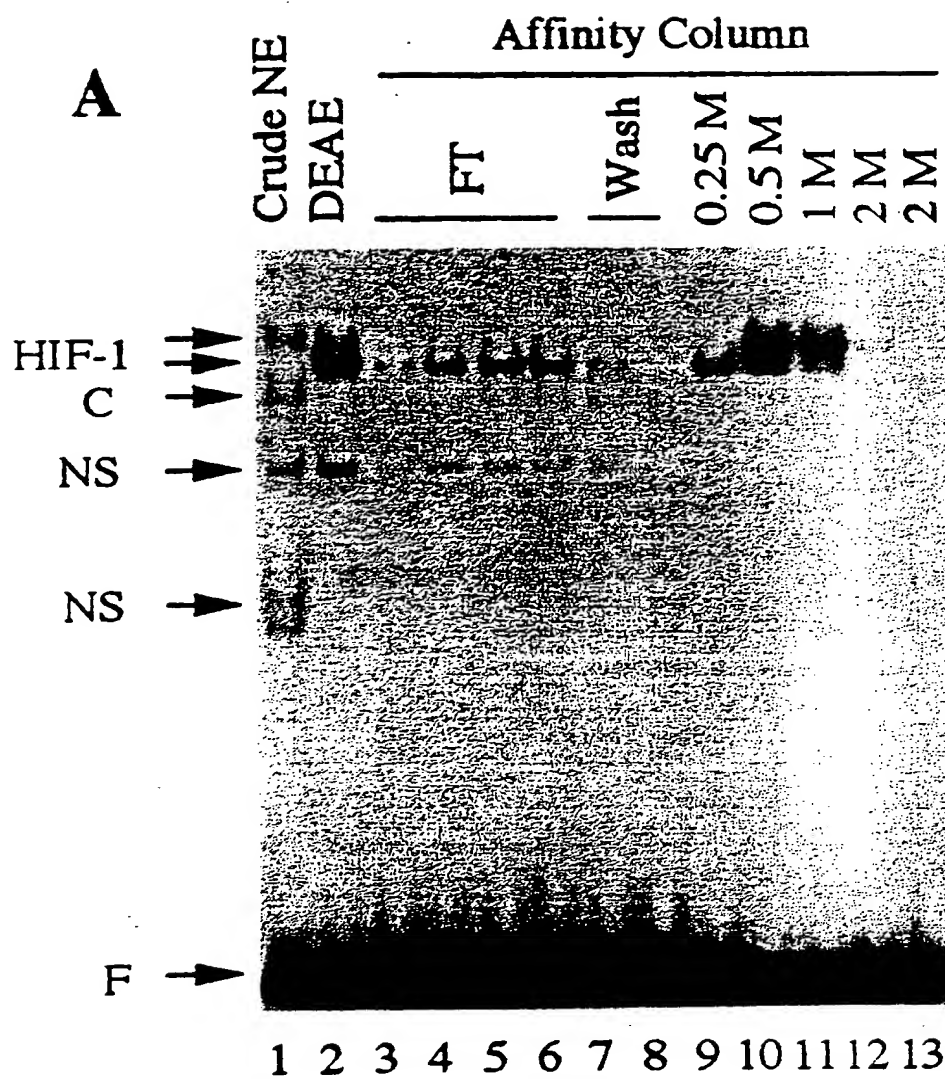


FIG. 3A

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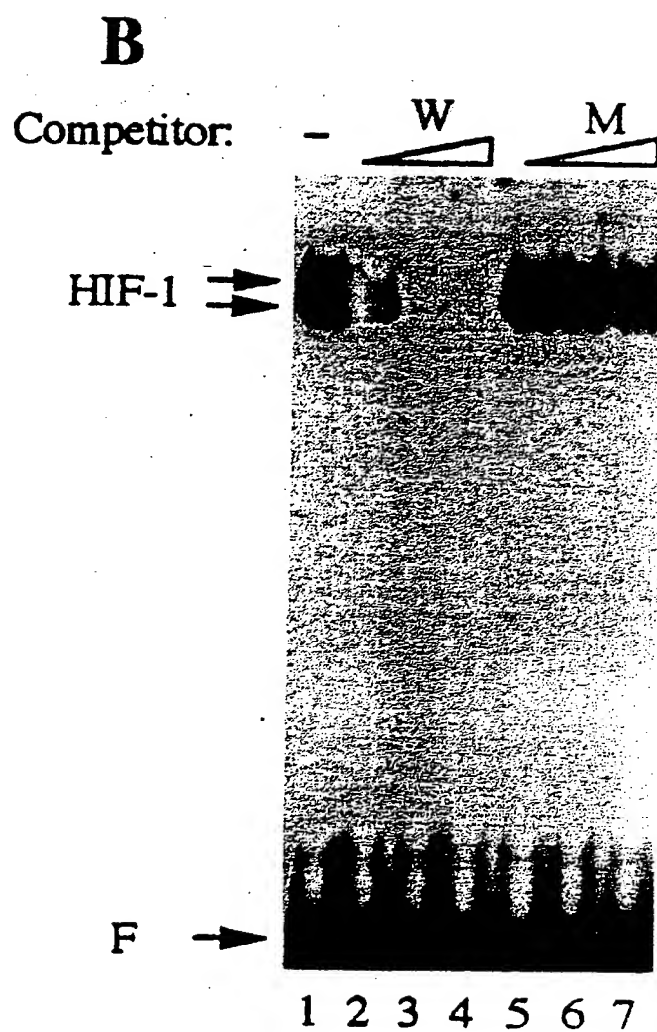


FIG. 3B

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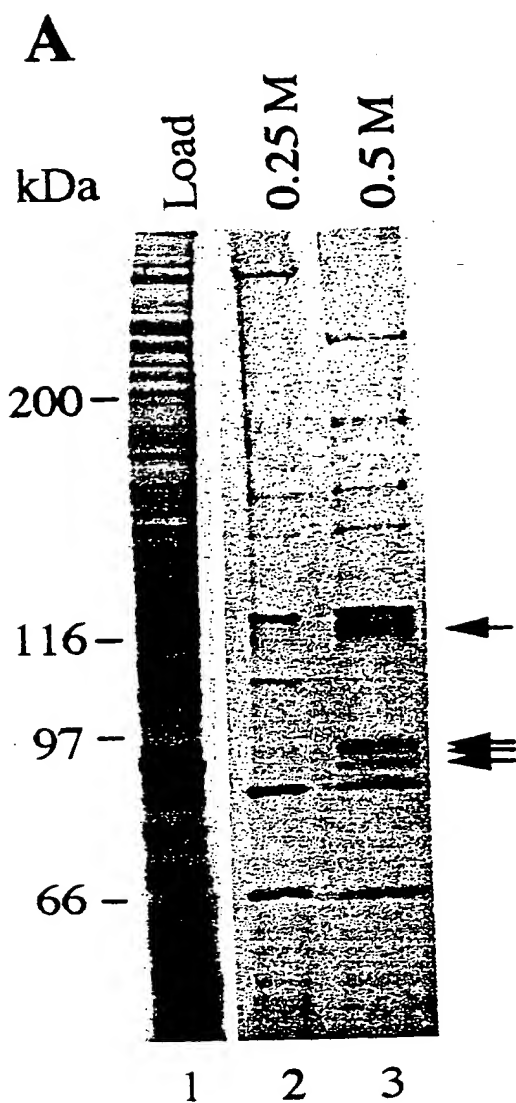


FIG. 4A

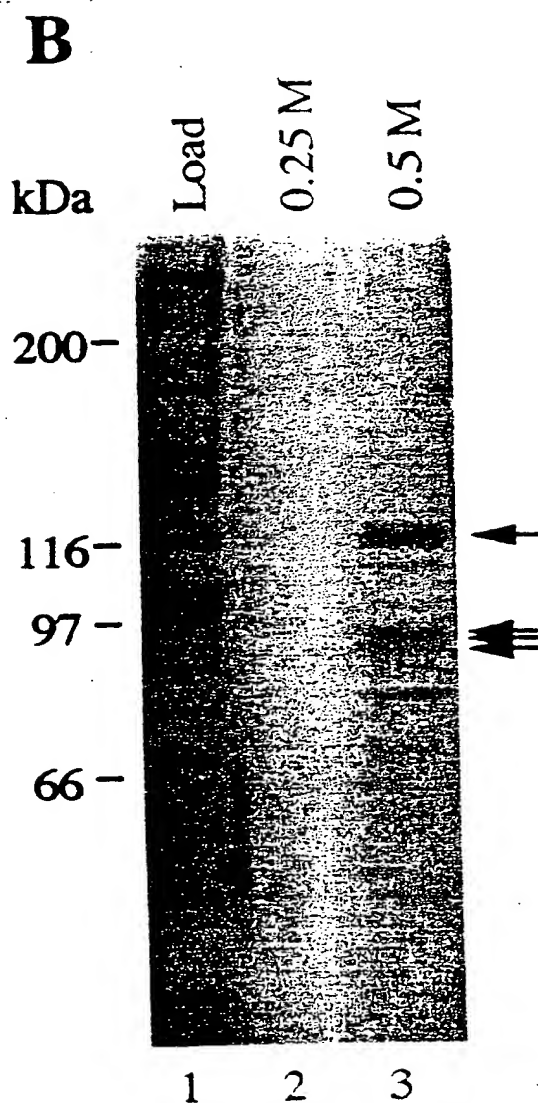


FIG. 4B

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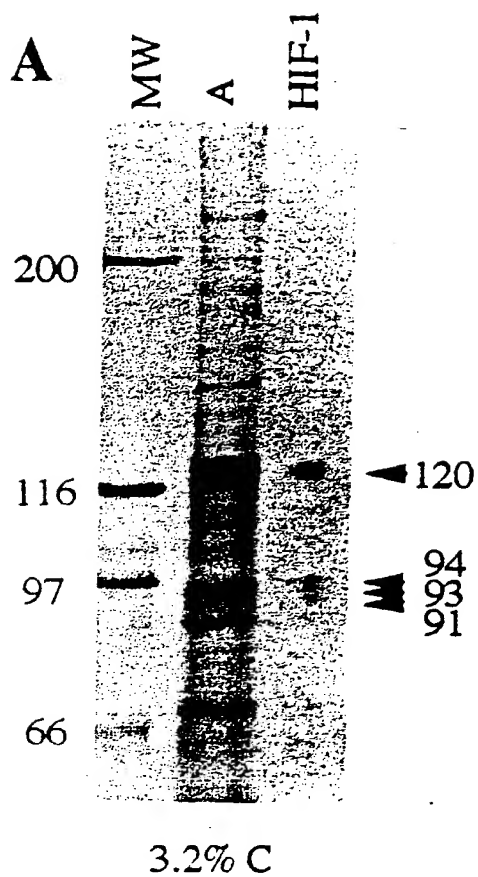


FIG. 5A

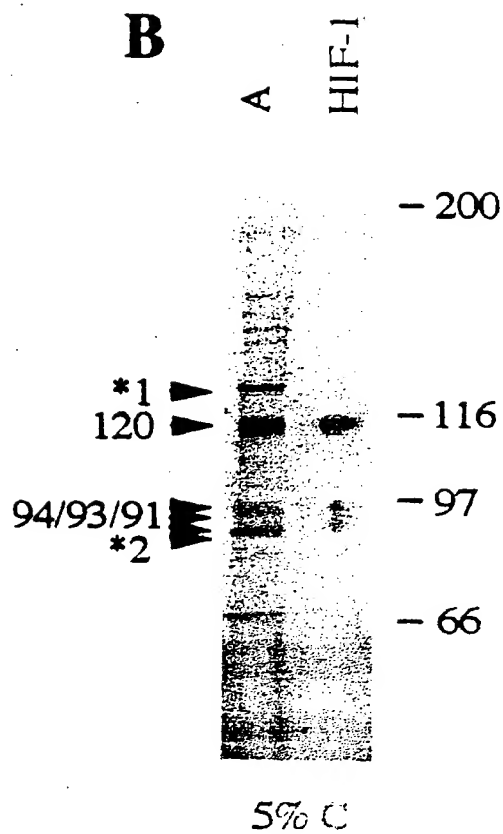
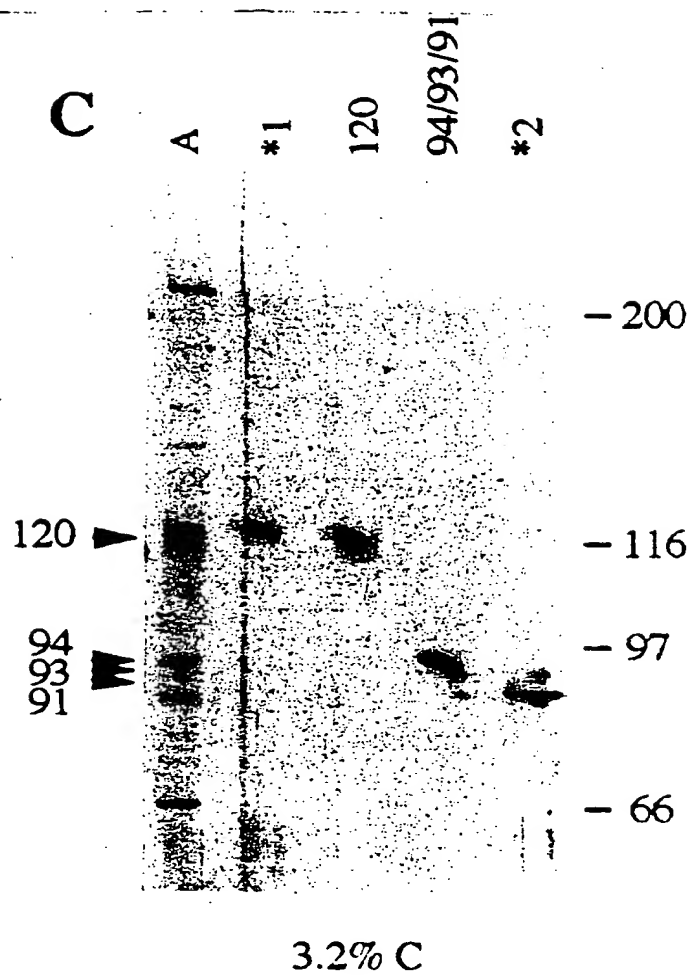


FIG. 5B

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**FIG. 5C**

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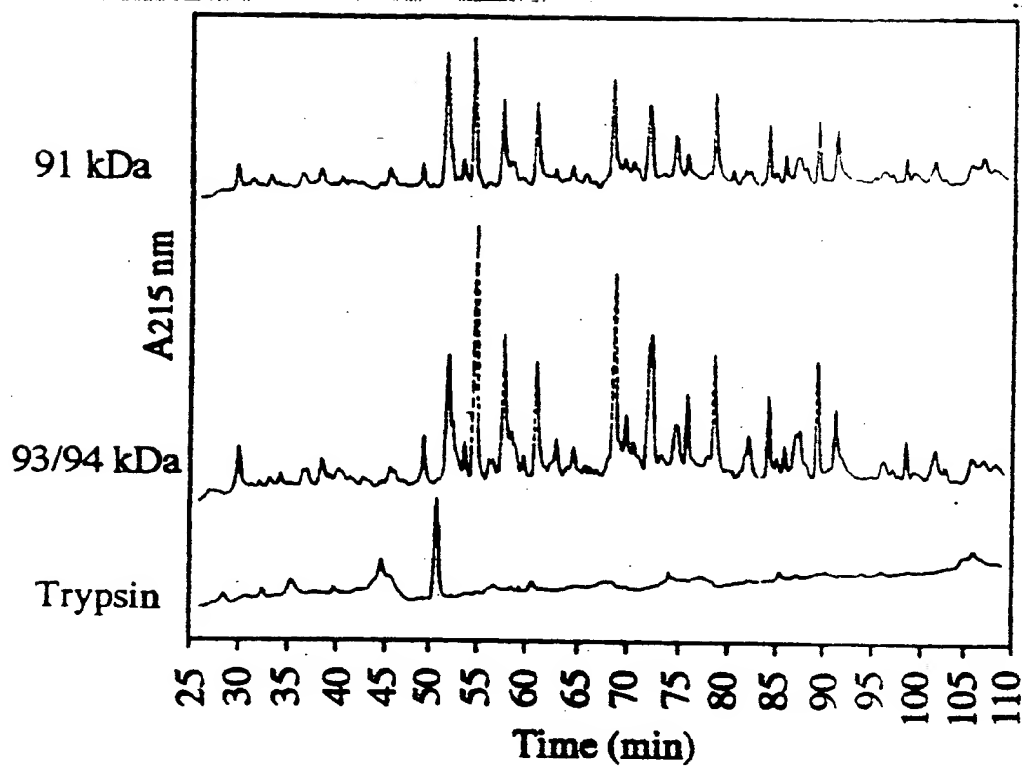


FIG. 6

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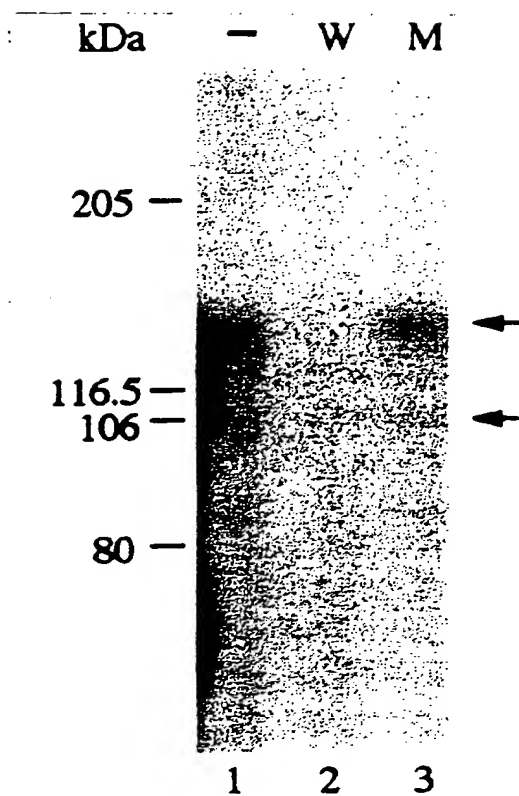


FIG. 7

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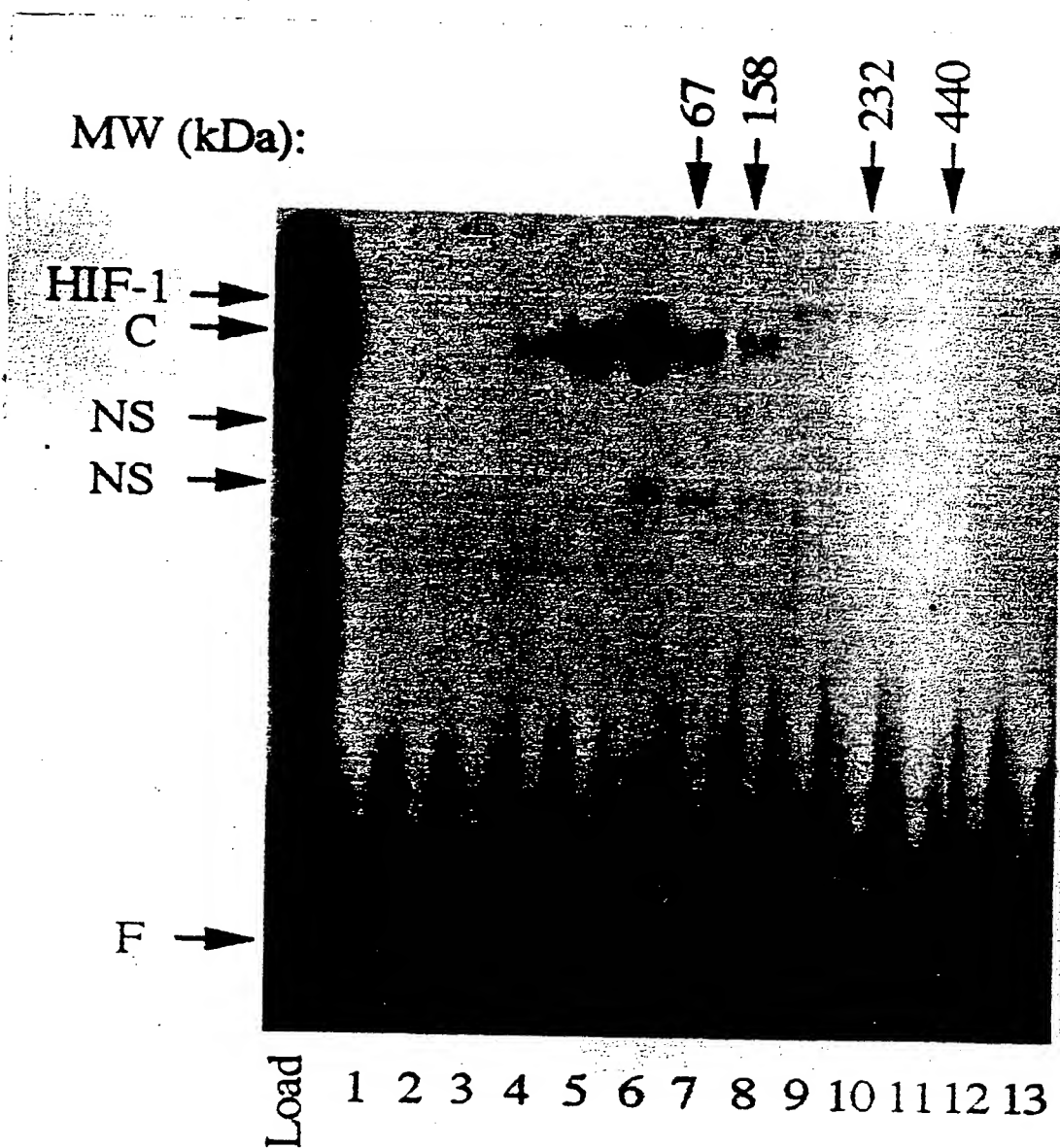


FIG. 8

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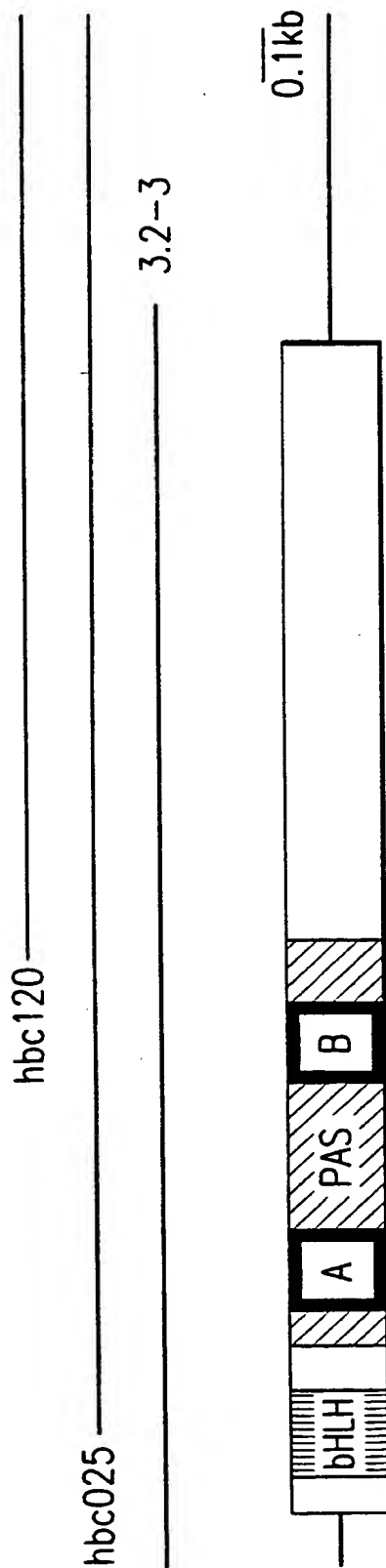


FIG. 9

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FIG. 10-1	FIG. 10-2	FIG. 10-3	FIG. 10-4
FIG. 10-5	FIG. 10-6	FIG. 10-7	FIG. 10-8

**FIG. 10**

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## FIG. 10-1

1  
1

62	AAG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAA	AAG
12	lys	ile	ser	ser	glu	arg	arg	lys	glu	lys
182	TCG	CAT	CTT	GAT	AAG	GCC	TCT	GTG	ATG	AGG
52	ser	his	leu	asp	lys	ala	ser	val	met	arg
302	TAT	TTG	AAA	GCC	TTG	GAT	GGT	TTT	GTT	ATG
92	tyr	leu	lys	ala	leu	asp	gly	phe	val	met
422	GIG	TTT	GAT	TTT	ACT	CAT	CCA	TGT	GAC	CAT
132	val	phe	asp	phe	thr	his	pro	cys	asp	his
542	AAG	TGT	ACC	CTA	ACT	AGC	CGA	GGA	AGA	ACT
172	lys	cys	thr	leu	thr	ser	arg	gly	arg	thr
662	TAT	AAG	AAA	CCA	CCT	ATG	ACC	TGC	TTG	GIG
212	tyr	lys	lys	pro	pro	met	thr	cys	leu	val
782	TTT	TCT	TAT	TGT	GAT	GAA	AGA	ATT	ACC	GAA
252	phe	ser	tyr	cys	asp	glu	arg	<u>ile</u>	<u>thr</u>	<u>glu</u>
902	CAT	GAT	ATG	TTT	ACT	AAA	GGA	CAA	GTC	ACC
292	his	asp	met	phe	thr	lys	gly	gln	val	thr
1022	CCA	CAG	TGC	ATT	GTA	TGT	GIG	AAT	TAC	GTT
332	pro	gln	cys	ile	val	cys	val	asn	tyr	val
1142	ACT	CAG	CTA	TTC	ACC	AAA	GTT	GAA	TCA	GAA
372	thr	gln	leu	phe	thr	lys	val	glu	ser	glu
1262	GAT	TTT	GGC	AGC	AAC	GAC	ACA	GAA	ACT	GAT
412	asp	phe	gly	ser	asn	asp	thr	glu	thr	asp
1382	CCA	TTA	CCC	ACC	GCT	GAA	ACG	CCA	AAG	CCA
452	pro	leu	pro	thr	ala	glu	thr	pro	lys	pro

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## FIG. 10-2

TCT CGA GAT GCA GCC AGA TCT CGG CGA AGT  
ser arg asp ala ala arg ser arg arg ser  
CTT ACC ATC AGC TAT TTG CGT GTG AGG AAA  
leu thr ile ser tyr leu arg val arg lys  
GTT CTC ACA GAT GAT GGT GAC ATG ATT TAC  
val leu thr asp asp gly asp met ile tyr  
GAG GAA ATG AGA GAA ATG CTT ACA CAC AGA  
glu glu met arg glu met leu thr his arg  
ATG AAC ATA AAG TCT GCA ACA TGG AAG GTA  
met asn ile lys ser ala thr trp lys val  
CTG ATT TGT GAA CCC ATT CCT CAC CCA TCA  
leu ile cys glu pro ile pro his pro ser  
TTG ATG GGA TAT GAG CCA GAA GAA CTT TTA  
leu met gly tyr glu pro glu glu leu leu  
ACA GGA CAG TAC AGG ATG CTT GCC AAA AGA  
thr gly gln tyr arg met leu ala lys arg  
GTG AGT GGT ATT ATT CAG CAC GAC TTG ATT  
val ser gly ile ile gln his asp leu ile  
GAT ACA AGT AGC CTC TTT GAC AAA CTT AAG  
asp thr ser ser leu phe asp lys leu lys  
GAC CAG CAA CTT GAG GAA GTA CCA TTA TAT  
asp gln gln leu glu glu val pro leu tyr  
CTT CGA AGT AGT GCT GAC CCT GCA CTC AAT  
leu arg ser ser ala asp pro ala leu asn

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## FIG. 10-3

GTGAAGACATCGCGGGACCGATTTCACC ATG

met

AAA GAA TCT GAA GTT TTT TAT GAG CTT GCT  
 lys glu ser glu val phe tyr glu leu ala  
 CTT CTG GAT GCT GGT GAT TTG GAT ATT GAA  
 leu leu asp ala gly asp leu asp ile glu  
 ATT TCT GAT AAT GTG AAC AAA TAC ATG GGA  
 ile ser asp asn val asn lys tyr met gly  
 AAT GGC CTT GTG AAA AAG GGT AAA GAA CAA  
 asn gly leu val lys lys gly lys glu gln  
 TTG CAC TGC ACA GGC CAC ATT CAC GTA TAT  
 leu his cys thr gly his ile his val tyr  
 AAT ATT GAA ATT CCT TTA GAT AGC AAG ACT  
 asn ile glu ile pro leu asp ser lys thr  
 GGC CGC TCA ATT TAT GAA TAT TAT CAT GCT  
~~gly arg ser ile tyr glu tyr tyr his ala~~  
 GGT GGA TAT GTC TGG GTT GAA ACT CAA GCA  
 gly gly tyr val trp val glu thr gln ala  
 TTC TCC CTT CAA CAA ACA GAA TGT GTC CTT  
 phe ser leu gln gln thr glu cys val leu  
 AAG GAA CCT GAT GCT TTA ACT TTG CTG GCC  
 lys glu pro asp ala leu thr leu leu ala  
 AAT GAT GTA ATG CTC CCC TCA CCC AAC GAA  
 asn asp val met leu pro ser pro asn glu  
 CAA GAA GTT GCA TTA AAA TTA GAA CCA AAT  
 gln glu val ala leu lys leu glu pro asn

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## FIG. 10-4

GAG GGC GCC GGC GGC GCG AAC GAC AAG AAA  
glu gly ala gly gly ala asn asp lys lys  
CAT CAG TTG CCA CTT CCA CAT AAT GTG AGT  
his gln leu pro leu pro his asn val ser  
GAT GAC ATG AAA GCA CAG ATG AAT TGC TTT  
asp asp met lys ala gln met asn cys phe  
TTA ACT CAG TTT GAA CTA ACT GGA CAC AGT  
leu thr gln phe glu leu thr gly his ser  
AAC ACA CAG CGA AGC TTT TTT CTC AGA ATG  
asn thr gln arg ser phe phe leu arg met  
GAT ACC AAC AGT AAC CAA CCT CAG TGT GGG  
asp thr asn ser asn gln pro gln cys gly  
TTC CTC AGT CGA CAC AGC CTG GAT ATG AAA  
phe leu ser arg his ser leu asp met lys  
TTG GAC TCT GAT CAT CTG ACC AAA ACT CAT  
leu asp ser asp his leu thr lys thr his  
ACT GTC ATA TAT AAC ACC AAG AAT TCT CAA  
thr val ile tyr asn thr lys asn ser gln  
AAA CCG GTT GAA TCT TCA GAT ATG AAA ATG  
lys pro val glu ser ser asp met lys met  
CCA GCC GCT GGA GAC ACA ATC ATA TCT TTA  
pro ala ala gly asp thr ile ile ser leu  
AAA TTA CAG AAT ATA AAT TTG GCA ATG TCT  
lys leu gln asn ile asn leu ala met ser  
CCA GAG TCA CTG GAA CTT TCT TTT ACC ATG  
pro glu ser leu glu leu ser phe thr met

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1502 CCC CAG ATT CAG GAT CAG ACA CCT AGT CCT  
492 pro gln ile gln asp gln thr pro ser pro  
1622 AAG TTG GAA TTG GTA GAA AAA CTT TTT GCT  
532 lys leu glu leu val glu lys leu phe ala  
1742 TTC CAG TTA CGT TCC TTC GAT CAG TIG TCA  
572 phe gln leu arg ser phe asp gln leu ser  
1862 GCT AAT GCC ACC ACT ACC ACT GCC ACC ACT  
612 ala asn ala thr thr thr thr ala thr thr  
1982 ACT AGT GCC ACA TCA TCA CCA TAT AGA GAT  
652 thr ser ala thr ser ser pro tyr arg asp  
2102 TCT GTC GCT TIG AGT CAA AGA ACT ACA GTT  
692 ser val ala leu ser gln arg thr thr val  
2222 GTA GGA ATT GGA ACA TTA TTA CAG CAG CCA  
732 val gly ile gly thr leu leu gln gln pro  
2342 ATT TTA ATA CCC TCT GAT TTA GCA TGT AGA  
772 ile leu ile pro ser asp leu ala cys arg  
2462 CTA CTG CAG GGT GAA GAA TTA CTC AGA GCT  
812 leu leu gln gly glu glu leu leu arg ala  
2605 CTACAATACTGCACAACTTGGTTAGTTCAATTTTTGAT  
2764 TTAAAAAATGCACCTTTTTATTIATTTATTTTTGGCTAG  
2923 TTTTACATAAATAATAATGCTTTGCCAGCAGTACGTTGGT  
3082 CTGGAACATGACATTGTTAATCATATAATAATGATTCTT  
3241 TCTGATGTTTCTATAGTCACTTTGCCAGCTCAAAAGAAA  
3400 AAAATCATGCATTCTTAGCAAATTGCCTAGTATGTTAA  
3559 CAGTAAATATCTTGTTTTTTCTATGTACATTGTACAAAT

FIG. 10-5

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TCC GAT GGA AGC ACT AGA CAA AGT TCA CCT  
ser asp gly ser thr arg gln ser ser pro  
GAA GAC ACA GAA GCA AAG AAC CCA TTT TCT  
glu asp thr glu ala lys asn pro phe ser  
CCA TTA GAA AGC AGT TCC GCA AGC CCT GAA  
pro leu glu ser ser ser ala ser pro glu  
GAT GAA TTA AAA ACA GTG ACA AAA GAC CGT  
asp glu leu lys thr val thr lys asp arg  
ACT CAA AGT CGG ACA GCC TCA CCA AAC AGA  
thr gln ser arg thr ala ser pro asn arg  
CCT GAG GAA GAA CTA AAT CCA AAG ATA CTA  
pro glu glu glu leu asn pro lys ile leu  
GAC GAT CAT GCA GCT ACT ACA TCA CTT TCT  
asp asp his ala ala thr thr ser leu ser  
CTG CTG GGG CAA TCA ATG GAT GAA AGT GGA  
leu leu gly gln ser met asp glu ser gly  
TTG GAT CAA GTT AAC TGA GCTTTTCTTAATTT  
leu asp gln val asn OPA  
CCCCCTTCTACTTAAATTTACATTAATGCTCTTTTTTTAGTA  
GGAGTTTATCCCTTTTTTCGAATTATTTTTTAAGAAGATGCC  
AGCCACAATTGCACAATATATTTTCTTAAAAAATACCAGC  
AAATGCTGTATGGTTTATTTAATTTAAATGGGTAAAGCCATT  
ACAATACCCATATGTAGTTGTGGGAAGTTTATGCTAATATTG  
TTTGCTCAAAATACAATGTTTGATTTTATGCACTTTGTGCG  
TTTTCATTCCCTTTTGCTCTTTGTGGTTGGATCTAACACTA

FIG. 10-6



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GAG CCT AAT AGT CCC AGT GAA TAT TGT TTT  
glu pro asn ser pro ser glu tyr cys phe  
ACT CAG GAC ACA GAT TTA GAC TTG GAG ATG  
thr gln asp thr asp leu asp leu glu met  
AGC GCA AGT CCT CAA AGC ACA GTT ACA GTA  
ser ala ser pro gln ser thr val thr val  
ATG GAA GAC ATT AAA ATA TTG ATT GCA TCT  
met glu asp ile lys ile leu ile ala ser  
GCA GGA AAA GGA GTC ATA GAA CAG ACA GAA  
ala gly lys gly val ile glu gln thr glu  
GCT TTG CAG AAT GCT CAG AGA AAG CGA AAA  
ala leu gln asn ala gln arg lys arg lys  
TGG AAA CGT GTA AAA GGA TGC AAA TCT AGT  
trp lys arg val lys gly cys lys ser ser  
TTA CCA CAG CTG ACC AGT TAT GAT TGT GAA  
leu pro gln leu thr ser tyr asp cys glu  
CATTCCTTTTTTTTGGACACTGGTGGCTCACTACCTAAAGC

TGTTCTTTAATGCTGGATCACAGACAGCTCATTTTTCTCAGT  
AATATAATTTTTTGTAAGAAGGCAGTAACCTTTTCATCATGAT  
AGTTACTCATGGAATATATTCTGCGTTTATAAACTAGTTT  
TACATAATATAGAAAGATATGCATATATCTAGAAGGTATGT  
TGTAAGTATATTAACCTTAAATGTTCTGCTTACCTGTGTG  
CTATTAACATCCTTTTTTTTCATGTAGATTTCATAATTGAG  
ACTGTATTGTTTTGTTACATCAAATAACATCTTCTGTGGA

FIG. 10-7

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TAT GTG GAT AGT GAT ATG GTC AAT GAA TTC  
tyr val asp ser asp met val asn glu phe  
TTA GCT CCC TAT ATC CCA ATG GAT GAT GAC  
leu ala pro tyr ile pro met asp asp asp  
TTC CAG CAG ACT CAA ATA CAA GAA CCT ACT  
phe gln gln thr gln ile gln glu pro thr  
CCA TCT CCT ACC CAC ATA CAT AAA GAA ACT  
pro ser pro thr his ile his lys glu thr  
AAA TCT CAT CCA AGA AGC CCT AAC GTG TTA  
lys ser his pro arg ser pro asn val leu  
ATG GAA CAT GAT GGT TCA CTT TTT CAA GCA  
met glu his asp gly ser leu phe gln ala  
GAA CAG AAT GGA ATG GAG CAA AAG ACA ATT  
glu gln asn gly met glu gln lys thr ile  
GTT AAT GCT CCT ATA CAA GGC AGC AGA AAC  
val asn ala pro ile gln gly ser arg asn  
AGICTATTTTATATTTTCTACATCTAATTTTAGAAGCCTGG

TTTTTGGTATTTTAAACCATTTGCATTGTCAGTACATCATT  
CATAGGCAGTTGAAAAATTTTACACCTTTTTTTTTCACA  
TTAAGAAGAAATTTTTTTTGGCCTATGAAATTTGTTAAAC  
GGCATTTTATTTGGATAAAATTCTCAATTCAGAGAAATCA  
GTATAAAGATATTTTIGAGCAGACTGTAAACAAGAAAAAA  
TAATTTTAGAAGCATTATTTTAGGAATATATAGTTGTCA  
AA

FIG. 10-8

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HIF-1 $\alpha$ :	(17)	RRKEKSRDAARSRR	SKESEVFYELAHQL	PLPHNVSSHLD	KASVMRLTISYLRVR	
ARNT:	(90)	ARENHSEI-ERRRR	NKMTAYTTELSDMV	PTCSALARKPD	KLTI LRMVSHMKSL	(24%)
SIM:	(1)	MKEKSKNAARTRR	EKENTEFCELA KLL	PLPAAITSQLD	KASVI RLTTSYLKMR	(62%)
AHR:	(27)	AEGIKSNPS-KRRHR	DRLNTELDRLASLL	PFPQDVINKLD	KL SVLR LSVTYLRAK	(35%)
CONSENSUS:		-----KS-----R-RR	-K1-----1-ELA-1L	P1P--1---LD	K-SV1RL2-SYL4-4	
		BASIC	HELIX	LOOP	HELIX	
ARNT:	(90)	ARENHSEIERRRR	-NKMTAYTELSDMV	PTCSALARKPD---	KLTI LRMVSHMKSL	
MI:	(205)	KKDNHNL I IRRRR	-FNINDRIKELGTLI	PKSNPDPMRWN --	KG TILKASVDYI RKL	(32%)
USF:	(200)	RRAQHNEVERRRR	-DKINNWI VQLSKII	PDCSMESTKSGQS	KGGILSKACDYIQEL	(38%)
L-MYC:	(286)	KRKNNHFLERRRR	-NDLRSRFLALRDQV	PTL-ASCSKAP---	KVVILSKALEYLQAL	(39%)
CP-1:	(223)	RKDSHKEVERRRR	ENINTA-INVLSDLL	PVR--ESSKAA--	--- ILARAAEYIQL	(37%)
CONSENSUS:		433-HNE1ERRRR	---1---1---LSD11	P-----K-----	K--IL-4A13YIQ-L	

FIG. 11

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HIF-1 $\alpha$ :	(106)	DGDMYISDNVNKYMGLTQFELTGHVSFDFTHPCDHEEMREML--THRNLVK	
ARNT:	(183)	TGRVVVSDSVTPVLNQPSQSEWFGSTLYDQVHPDDVDKLREQL--STSENALT	(29%)
AHR:	(130)	DALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLHWALNPSQCT	(23%)
SIM:	(97)	DGKIMYISETASVHLGLSQVELTGNISIFEYIHNYDQDEMNAI--SLHPHINQ	(37%)
PER:	(286)	DGIVLYTTPSITDVLGYPRDMWLGRSFIDFVHLKDRATFASQI--TTGIPIAE	(22%)
kinA:	(24)	NGRIIYISANSKHLGLYLQGEMIGSFLKTFLHEEDQFLVESYFYNEHHLMPCCT	(26%)
CONSENSUS:		DG-11YIS-21---LG--Q-E11G-S1-311H--D---1---L--2-----	
HIF-1 $\alpha$ :	(249)	DMKFSYCDERITELMGYEPEELLGR-SIYEYHALSDHLLTK--THHDMFTKGQ	
ARNT:	(370)	EGIFTFVDHRCVATVGYQPQELLGK-NIVEFCHPEDQQLLRDSFQQVVKLKGQ	(35%)
AHR:	(288)	DFTPIGCCDAKGRIVLGYTEAELCTRGSGYQFIHAADMLYCAE-SHIRMIKTGE	(31%)
SIM:	(263)	DMKLIFFDARVSQLTGYEPQDLIEK-TLYQYIHAADIMAMRC-SHQILLYKGG	(39%)
PER:	(336)	TGIISHVDSAAVSALGYLPQDLIGR-SIMDFYHHEDLSVMKE-TYETVMKKGQ	(33%)
kinA:	(288)	WVFMNESGISLFEATYE--DLICK-NIYDQLHPCDHEDVKERIQNIAEQKTE	(19%)
CONSENSUS:		31-1---D-4-----1GY-P-3L1G4-2TY3--H--D---143-2---11-KGQ	

FIG. 12

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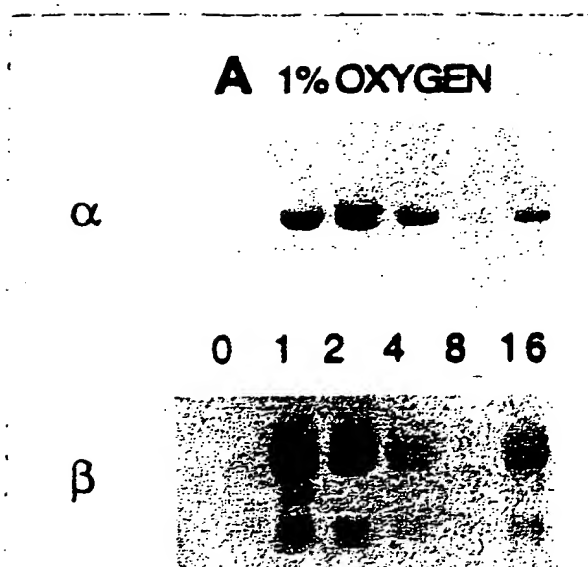


FIG. 13A

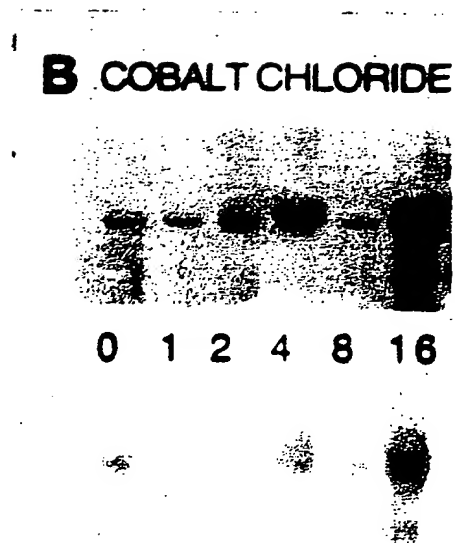


FIG. 13B

**C DESFERRIOXAMINE**

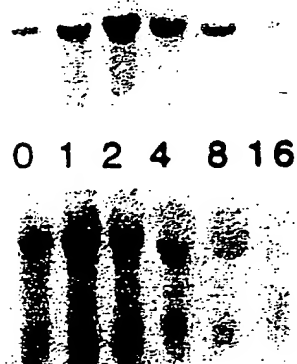


FIG. 13C

**D POST-HYPOXIA**

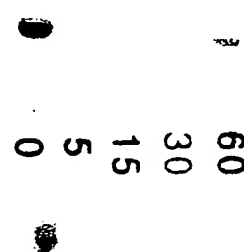


FIG. 13D

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**E 3'-UNTRANSLATED SEQUENCES**

2568 CU AUUUA UA  
2656 UA AUUUA CA  
2731 GU AUUUA AA  
2781 UU AUUUA UU  
2785 UU AUUUA UU  
3138 UU AUUUA AA  
3156 CC AUUUA CA  
3203 GC AUUUA UU

**FIG. 13E**

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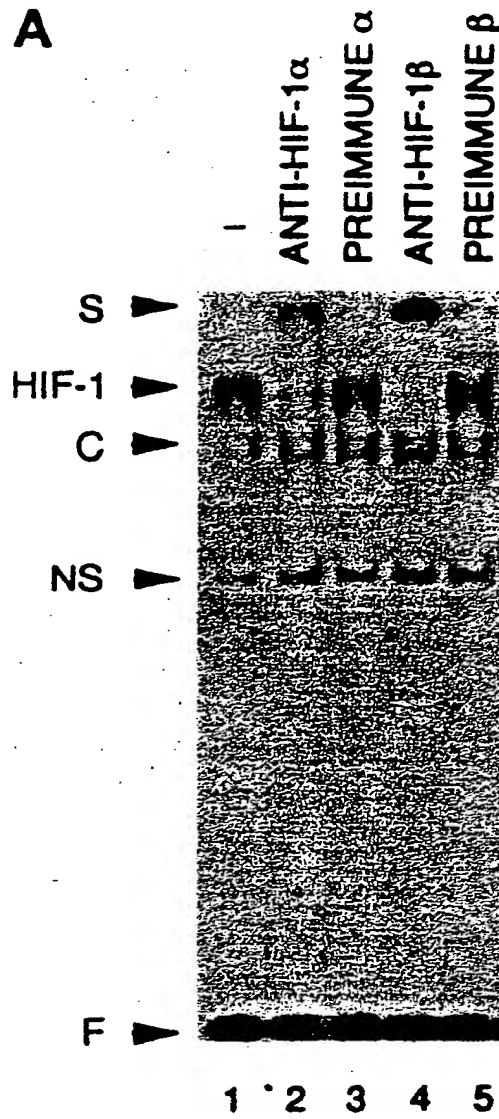


FIG. 14A

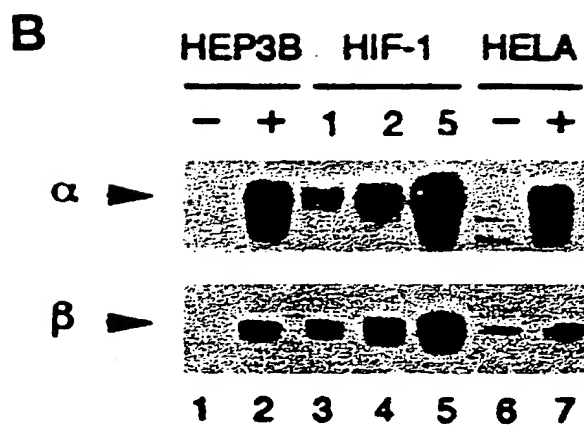


FIG. 14B

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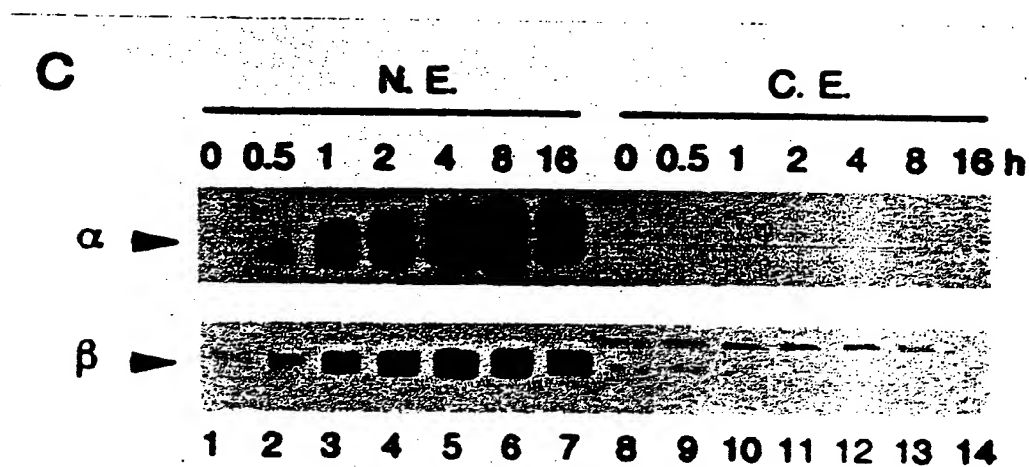


FIG. 14C

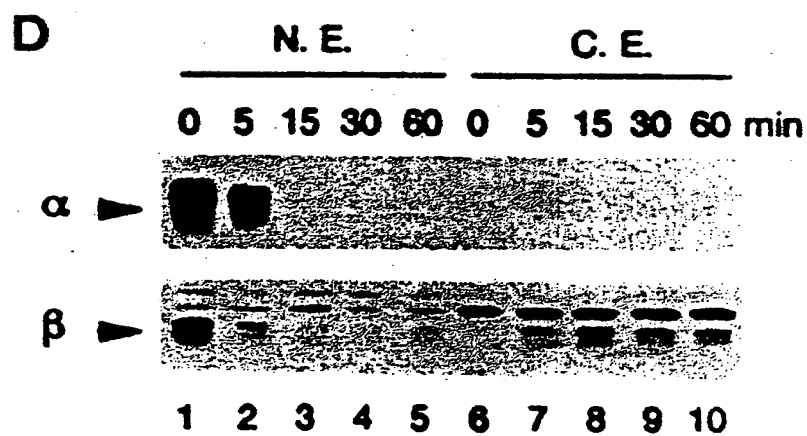


FIG. 14D



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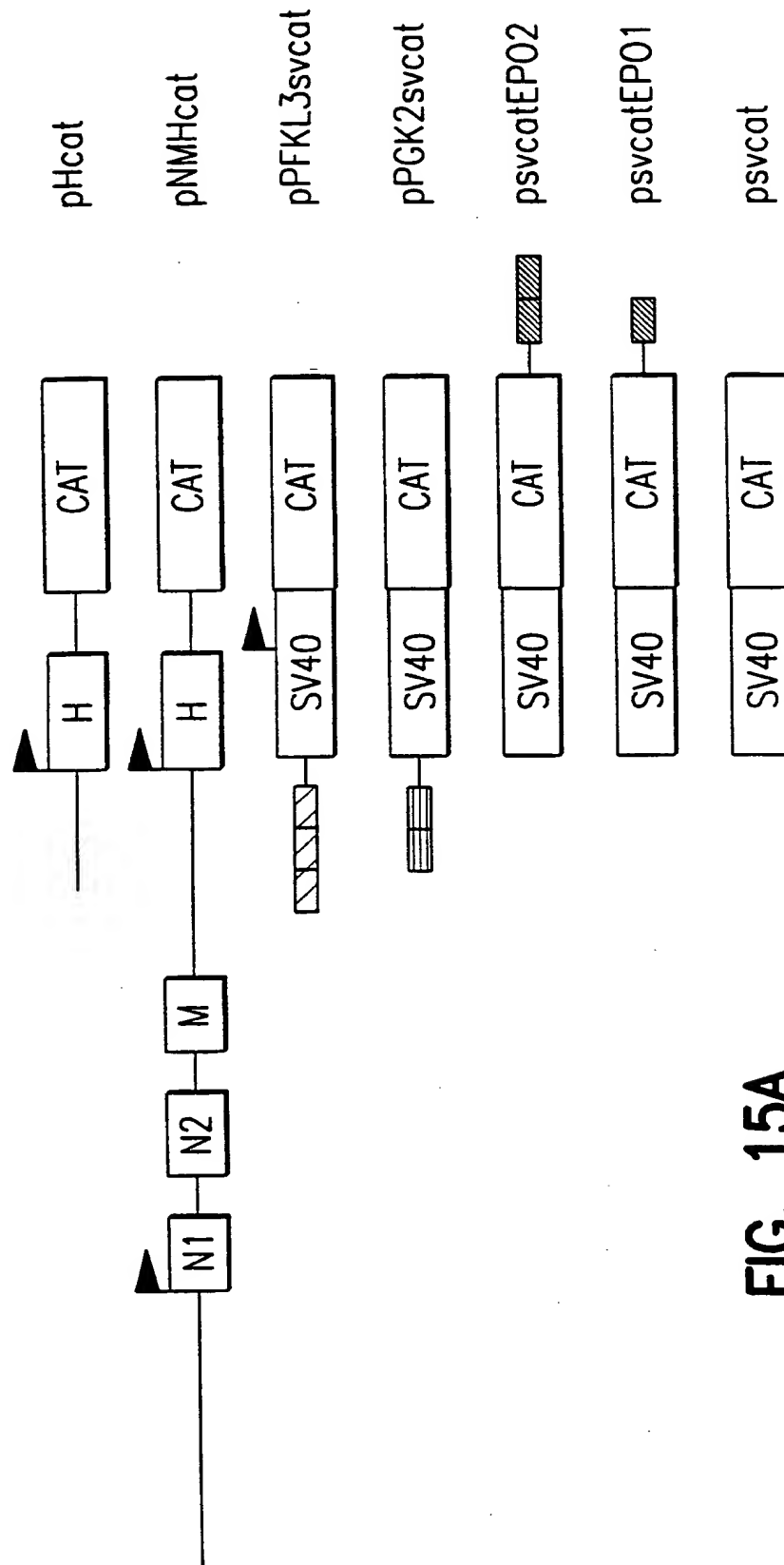


FIG. 15A

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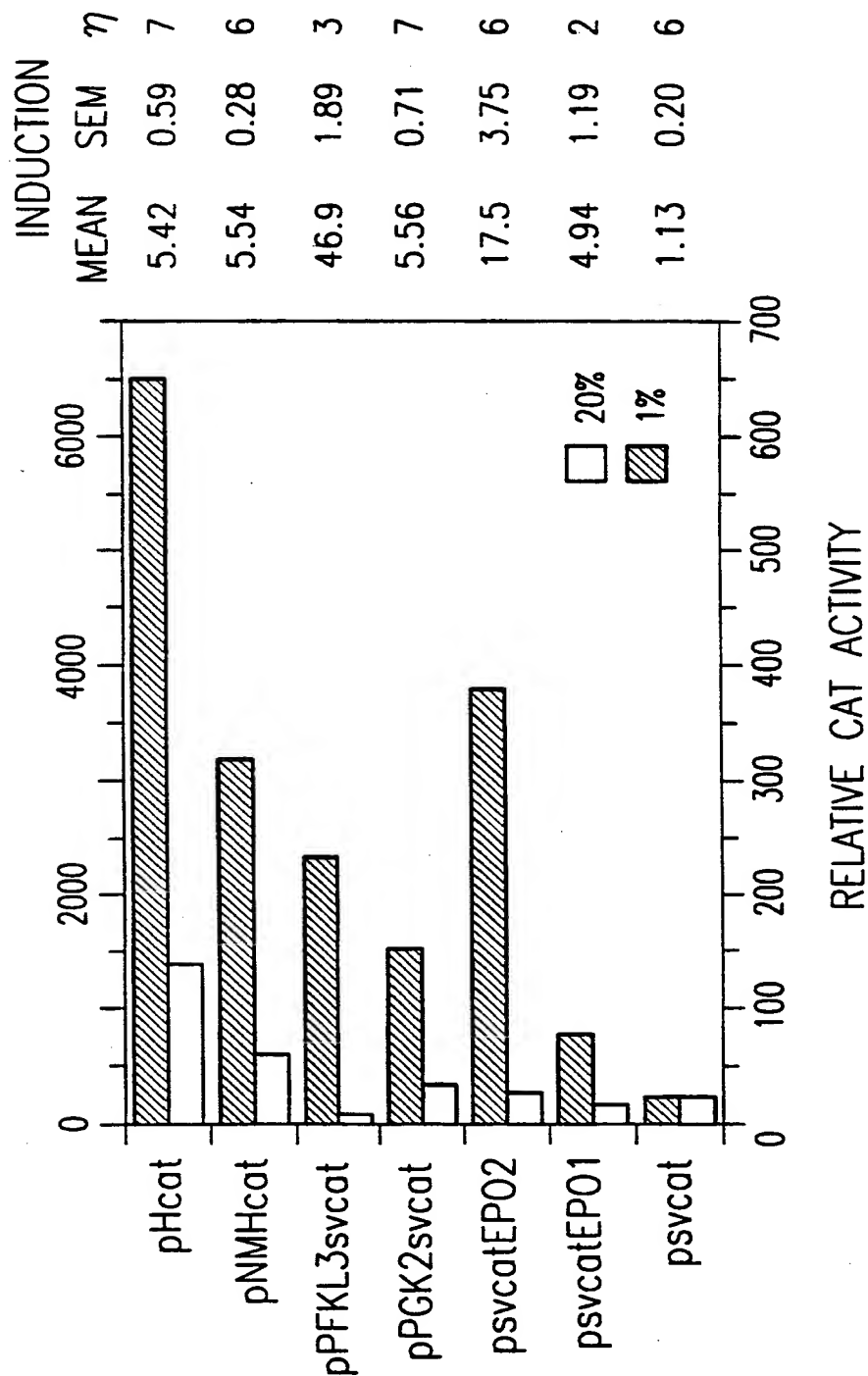


FIG. 15B

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AMINO-TERMINAL AMINO-ACID SEQUENCE (ENCOMPASSING BASIC DOMAIN)  
OF WILD-TYPE AND DOMINANT-NEGATIVE-MUTANT FORMS OF HIF-1 $\alpha$

HIF-1 $\alpha$  : 1/MEGAGGANDKKISSERRKEKSRDAARSRR/30

HIF-1 $\alpha$   $\Delta$  NB : 1/MEGIAG-----SRR/30

HIF-1 $\alpha$   $\Delta$  NB  $\Delta$  AB : 1/MEGIAG-----SRR/30

CARBOXY-TERMINAL AMINO-ACID SEQUENCE OF WILD-TYPE AND  
DOMINANT-NEGATIVE-MUTANT FORMS OF HIF-1 $\alpha$

HIF-1 $\alpha$  : 390/LKKEPDALT/400/820/RALDQVN/826

HIF-1 $\alpha$   $\Delta$  NB : 390/LKKEPDALT/400/820/RALDQVN/826

HIF-1 $\alpha$   $\Delta$  NB  $\Delta$  AB : 390/LKIQI-----/395

**FIG. 16**

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10251

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07K 14/00; C07H 21/04; C12N 1/12, 1/20, 5/00, 15/63

US CL : 435/240.1, 252.1, 320.1; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 252.1, 320.1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dejgaard et al. Identification, Molecular Cloning, Expression and Chromosome Mapping of a Family of Transformation Upregulated hn RNP-K Proteins Derived by Alternative Splicing, Journal of Molecular Biology. 11 February 1994, Vol. 236, No.1, pages 33-48, especially abstract on page 33 and Figure 5 on page 40.	1-12 and 16-18
A	Benjamin et al. Activation of the Heat Shock Transcription Factor by Hypoxia in Mammalian Cells. Proceedings of the National Academy of Sciences USA. August 1990, Vol. 87, No. 16, pages 6263-6267.	1-12 and 16-18

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*& document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 AUGUST 1996

Date of mailing of the international search report

02 OCT 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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Telephone No. 703 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/10251

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Wang et al. Hypoxia-Inducible Factor 1 is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O <sub>2</sub> Tension. Proceedings of the National Academy of Sciences USA. 06 June 1995, Vol. 92, No. 12, pages 5510-5514.	1-12 and 16-18
A, P	Norris et al. Hypoxia-Induced Protein Binding to O <sub>2</sub> -Responsive Sequences on the Tyrosine Hydroxylase Gene. The Journal of Biological Chemistry. 06 October 1995, Vol. 270, No. 40, pages 23774-23779.	1-12 and 16-18
A	Fujiwara et al. Expressed Sequence Tag (EST) GenBank Accession Numbers D56430, D53682, R71408, T32121, HS14513, T32145, T35966, M85743, R71117, T32012, and Q60265. GenBank. 30 May 1995, see sequence alignments.	3-12, 16, and 18

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10251

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-12 and 16-18
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

MPSEARCH for SEQ ID NO:1, 2, and 4; APS, MEDLINE, BIOSIS, CAPLUS, WPIDS

search terms: hypoxia, transcription factor, mammalian, inducible

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

PCT Rule 13.1 states that the "...international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ('requirement of unity of invention') ."

Group I, claims 1-2 and 17, drawn to polypeptides (first product).

Group II, claims 3-12, 16, and 18, drawn to polynucleotide sequences which encode an animal polypeptide, vectors encoding such sequences, and host cells containing such vectors (second product).

Group III, claims 13-15, drawn to antibodies (third product).

Group IV, claims 19 and 21, drawn to a method of using antibodies or other binding reagent (first method of using the third product).

Group V, claim 20, drawn to a method of detecting a nucleotide sequence (first method of using the second product).

Group VI, claims 22-27, drawn to a method of treatment using nucleotides (second method of using the second product).

Group VII claims 28-32, drawn to a method of treatment using antisense (third method of using the antisense of the second product).

Group VIII, claim 33, drawn to a pharmaceutical composition comprising a polypeptide (fourth product).

Group IX, claim 34, drawn to a pharmaceutical comprising a nucleotide sequence (fifth product).

Group X, claim 35, drawn to a pharmaceutical composition comprising an inhibitory nucleotide sequence (sixth product).

Unity of invention does not exist because the products of Groups I, II III, VIII, IX, and X do not share a special technical feature since they are drawn to polypeptides, nucleotide sequences, antibodies, and pharmaceutical compositions comprising a polypeptide, nucleotide sequence, or the antisense of the nucleotide sequence, which are chemically and structurally distinct. Groups IV, V, VI, and VII are drawn to different methods of using the distinct products of Group II and III.

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